Pan-Family Assays for Rapid Viral Screening: Reducing Delays in Public Health Responses During Pandemics

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Summary:

SARS-CoV-2 has highlighted deficiencies in the testing capacity of many countries during the early stages of pandemics. Here we describe a strategy utilizing pan-family viral assays to improve early accessibility of large-scale nucleic acid testing.

Abstract

Background: COVID-19 has highlighted deficiencies in the testing capacity of many developed countries during the early stages of pandemics. Here we describe a strategy utilizing pan-family viral assays to improve early accessibility of large-scale nucleic acid testing.

Methods: Coronaviruses and SARS-CoV-2 were used as a case-study for assessing utility of pan-family viral assays during the early stages of a novel pandemic. Specificity of a pan-coronavirus (Pan-CoV) assay for a novel pathogen was assessed using the frequency of common human coronavirus (HCoV) species in key populations. A reported Pan-CoV assay was assessed to determine sensitivity to 60 reference coronaviruses, including SARS-CoV-2. The resilience of the primer target regions of this assay to mutation was assessed in 8893 high-quality SARS-CoV-2 genomes to predict ongoing utility during pandemic progression.

Results: Due to common HCoV species, a Pan-CoV assay would return false positives for as few as 1% of asymptomatic adults, but up to 30% of immunocompromised patients with respiratory disease. Half of reported Pan-CoV assays identify SARS-CoV-2 and with small adjustments can accommodate diverse variation observed in animal coronaviruses. The target region of one well established Pan-CoV assay is highly resistant to mutation compared to species-specific SARS-CoV-2 RT-PCR assays.

Conclusions: Despite cross-reactivity with common pathogens, pan-family assays may greatly assist management of emerging pandemics through prioritization of high-resolution testing or isolation measures. Targeting highly conserved genomic regions make pan-family assays robust and resilient to mutation. A strategic stockpile of pan-family assays may improve containment of novel diseases prior to the availability of species-specific assays.

Keywords: Viral Screening, Pan-Family Assays, SARS-CoV-2

Introduction

During the early stages of the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) pandemic many countries exhibited an extreme shortage of nucleic acid test kits. This resulted in a weeks to months-long period where testing could only be performed in a limited capacity at select test centers, with a focus on symptomatic patients with a history of travel or association with a known case. The inability to perform extensive testing was particularly impactful in the current pandemic due to the greater than normal infectivity of asymptomatic patients, placing a greater burden on molecular diagnostic tools for identifying and containing disease spread[1]. The precise cause of this shortage has not yet been fully investigated but is likely a combination of the unprecedented global demand for test reagents and equipment, disruption to supply chains caused by the pandemic and regulatory restrictions limiting the ability of some nations to expand test capacity[2]. These shortcomings highlight that the current testing infrastructure and capacity expansion strategies are not rapid enough to counter disease spread during the early stages of some pandemics.

In response to SARS-CoV-2, governments will likely invest in a more extensive and agile network of testing equipment, stockpile test reagents and consumables and streamline test certification protocols. While this may enhance the speed at which testing capacity can be increased, there will still be a vital period between when a novel pathogen emerges and when species-specific tests are widely available at high capacity. This inability to perform extensive screening can hamper efforts to contain disease spread, particularly in the case of outbreaks with asymptomatic transmission where isolation based on symptoms is insufficient. A strategy which may allow for less-restricted screening for novel pathogens during this time period is the use of pre-emptively developed and distributed pan-family assays: molecular diagnostic tests targeted at a family of viruses rather than a single species.

Pan-family assays targeting viruses on the family level have been previously developed for research applications. Several reverse-transcription PCR (RT-PCR) assays targeting the highly conserved coronavirus RNA-dependent RNA polymerase gene with a pool of degenerate primers have been previously described [3-6]. These assays have been used to detect and amplify genomic material of unknown coronaviruses for genomic sequencing [7] and perform retrospective analysis of clinical samples to assess prevalence of coronavirus infection[8].

To reduce the likelihood of severe test shortages in future pandemics, we propose a proactive strategy involving the large-scale stockpile and distribution of approved and clinically validated pan-family diagnostic test kits **(supplementary figure 1).** These kits would serve as a bridging tool to allow for improved identification of infected individuals while species-specific tests are developed, distributed and certified by regulatory bodies. By ensuring there is a robust supply of test kits restrictions on testing eligibility could be greatly relaxed, possibly preventing escalation to pandemic state. Furthermore, due to the less restricted targets of pan-family assays they may be applied more routinely during local epidemics without the need to optimize for local pathogens.

Here we assess, using coronaviruses and SARS-CoV-2 as a case study, whether pan-family targeted assays are a practical tool for high-throughput screening of infection during the early stages of viral outbreaks. We consider the frequency of common viral species, the capability of reported pan-family assays to accommodate observed variation and the mutation rate of the pan-family assay target regions. Given the significant global economic and social disruption caused by the SARS-CoV-2 pandemic, we argue that despite some limitations in

the specificity and sensitivity of pan-family assays, a strategic stockpile is prudent as a first-line mass-screening technique.

Methods

Review of common HCoV epidemiolocal characteristics

A search using the terms “HCoV” “epidemiology” and “asymptomatic” was performed in PubMed to identify studies describing HCoV infection characteristics. Studies highlighting the following aspects of HCoV infection were selected and summarized; frequency in children or the elderly, frequency in asymptomatic individuals, frequency in large study cohorts and frequency during local epidemics.

Validation and modification of a reported Pan-CoV assays

Coronavirus-family reference sequences were identified and downloaded from the NCBI Virus database[9]. Sequences for a total of 60 species were identified **(Supplementary Table 1)**, including the 7 species known to infect humans (HCoV-229E, HCoV-NL63, HCoV-HKU1 and HCoV-OC43, SARS-CoV, MERS-CoV and SARS-CoV-2). Pan-coronavirus assay primer targets were derived from four reported assays[3-6] and aligned with the SARS-CoV-2 reference genome (NC_045512) to assess consensus between sequences using the Clustal Omega Multiple Sequence Alignment tool[10].

The nucleic acid sequence and predicted protein sequence for each species was aligned and assessed for identity with Pan-CoV primer targets using the software package Geneious Prime 2020.1.2 (https://www.geneious.com). Primers were modified to accommodate observed nucleic acid variation as well as nucleic acid sequences expected from the observed protein variation.

Assessment of the Pan-CoV primer target site mutation rate

Mutation frequency of the Pan-CoV target site was assessed using 8,899 high coverage (<1% N calls and <0.05% unique amino acid mutations) full length (>29,000 base pairs) SARS-CoV-2 genomes downloaded from the GISAID EpiCoV database[11]. Analysis was performed with the R Biostrings package[12] to identify mismatches in the primer target sites. Three samples were excluded due to missing sequence data in the primer target region.

Results

Specificity of a Pan-CoV assay to a novel pathogen – impact of common HCoV species

A key factor in the utility of pan-family assays is the frequency of common species of the target family in the general population, as these will interfere with the specificity of an assay for the novel pathogen. For the coronavirus family there are 4 endemic and common species (HCoV-229E, HCoV-NL63, HCoV-HKU1 and HCoV-OC43). These typically cause mild disease but can lead to severe or fatal infections in frail or immunosuppressed patients. It remains to be seen whether the current SARS-CoV-2 pandemic will persist at low levels in the general population following disease control through social distancing, therapeutic measures or acquisition of herd immunity[13]. Non-endemic zoonotic coronavirus species (SARS-CoV, MERS-CoV), while highly pathogenic, would not affect the specificity of a Pan-CoV assay for a novel pathogen as they are not observed at a significant frequency. However, Pan-CoV assay sensitivity to these pathogens is important as they are informative of species with zoonotic potential.

Table 1 summarizes studies reporting the prevalence of common HCoV infection[8, 14-24]. HCoV infections are typically detected in 2-10% of patients exhibiting acute respiratory illness (ARI), although during local HCoV epidemics frequency of infection may be as high as 30% of patients. Additionally, HCoV infections display a seasonality, typically observed in winter months. Children, both experiencing ARI and asymptomatic have a high occurrence of HCoV infection (4-10%). In asymptomatic adults HCoV infection is less well studied, but reported values are lower at ~1%. Studies of seroprevalence of HCoV antibodies indicate that virtually all individuals have had prior exposure to at least one species, with first exposure common in childhood[25]. This high frequency of childhood infection may explain the lower prevalence of asymptomatic infection in adults due to a partial acquired immunity[26], though limited data exists describing the immunizing effect of HCoV infection[27].

Considering these observed frequencies of common HCoV species, a Pan-CoV assay would be expected to be sufficiently specific for a novel pathogen to allow for broad population screening, wherein a positive test is used to guide infection controls such as self-isolation. The screening specificity is strongest in asymptomatic adults where only ~1% of the population would be expected to return a false positive due to HCoV infection.

Sensitivity of Pan-CoV assays to novel pathogen – performance of reported assays on observed coronavirus species from animal populations

Figure 1 compares the target sequences of reported Pan-CoV assays with the SARS-CoV-2 reference genome[3-6]. Only 2 of the 4 reported assays tested, the Moes/Vijgen (2005) update of the Stephensen (1999) assay and the recent Hu (2018) assay, accommodate the SARS-CoV-2 genome without any primer-template mismatches. While these mismatches may not prevent detection, they are undesirable as they may reduce reaction sensitivity or co-operate with future mutations to reduce test sensitivity[28].

In contrast to the other reported Pan-COV assays, the Moes/Vijgen assay (MPC) has a detailed protocol for analysis of respiratory pathogens in human samples[4]. To better predict the sensitivity of this assay to novel pathogens, we compared the MPC primer sequences to the respective primer target sites in the genomes of 60 coronavirus species **(Supplementary Table 1)**. Many of these species were reported following the development of the MPC assay, and as such are informative of the capacity of existing tests to detect novel pathogens.

Of the coronavirus reference sequences investigated, 33% (20/60) contained a mismatch between the MPC degenerate primer target and the viral genome, suggesting the MPC assay would be sub-optimal for detection of these species **(Supplementary Table 1)**. Only 7% (4/60) of species contained a genomic variant resulting in a change to the amino acid motifs encoded by the target region. (**Supplementary Table 2)**. This highlights the importance of accommodating unobserved but likely variation, such as silent mutations, when designing

pan-family assays. Redesigned primers accommodating the observed sequence data are detailed in **Supplementary Table 3.**

Resistance of the MPC target region to mutation

Another application of a robust pan-family assay is as a fallback to protect against reduction in test sensitivity of species-specific assays due to mutation of the viral pathogen during pandemic progression. To assess the resilience of the MPC assay to mutation-derived errors, we assessed mutation frequency of the MPC target sites in 8,893 high-coverage SARS-CoV-2 genomes. Only a single sample with a mutation in the MPC target region was observed, EPI_ISL_414596, translating to an observed mutation rate of 0.01%. This is an order of magnitude lower than the observed mutation rate for the CDC primer regions (0.4-2.58%) and the China CDC primer regions (0.74-16.19%) in the same dataset as reported on the GISAID platform[11]. These results indicate that the MPC target region is robust to mutation and may provide ongoing value in the case of emergence of a viral strain with mutations in the target regions of high specificity assays.

Discussion

Nucleic acid testing for the identification of infected individuals is one the most valuable tools for controlling pathogen spread, especially for diseases with asymptomatic transmission. The recent SARS-CoV-2 pandemic has revealed deficiencies in the testing capacity of many countries, resulting in a period where testing was highly restricted, preventing optimal disease containment. The use of family-wide viral assays may reduce the time to mass screening by allowing tests to be developed and distributed prior to pathogen emergence. Here we demonstrate that a Pan-CoV test can be an effective tool for management of novel coronavirus pathogens. These results may also be more broadly

applicable for the implementation of pan-family assays for the detection of pathogens from other viral families.

Coronaviruses are a useful framework for assessing the utility of pan-family testing as several HCoV species are common globally. While common HCoV species interfere with specific detection of a novel pathogen, even without confirmatory species-specific testing a Pan-CoV test allows for a large majority (>90%) of individuals, particularly asymptomatic adults, to avoid self-isolation measures with a negative test. However, greater caution must be taken in populations with a higher HCoV infection rate such as children, symptomatic patients with comorbidities, or populations experiencing a local HCoV epidemic.

This limitation can be mitigated through multiple strategies, most simply application of pan-family assays as a first-line screen prior to confirmatory analysis with a species-specific test. This would allow short-supply specific tests to be reserved as a confirmatory tool, enabling a higher-throughput screen of large populations at ports-of-entry or for contact tracing during the early stages of pandemic. An alternative strategy not reliant on the presence of a specific test would be the addition of a second exclusionary reaction targeting known common species[29], though this may not be an effective tool where a novel pathogen is highly genetically similar to a common species and may be overly burdensome for high-throughput population screening.

During the initial roll-out of SARS-CoV-2 testing kits by the CDC, several laboratories experienced disruptions due to faulty testing kits[30]. Pan-family assays, developed prior to an outbreak, would have a relaxed timeframe for test development, ensuring best-practice validation, quality control procedures, regulatory certification and laboratory accreditation can be achieved. Pan-family assays may also have additional ongoing utility as a fallback in

the case of quality control issues or disease mutations compromising the accuracy of specific assay detection[31]. By targeting highly conserved regions and accommodating silent mutations, pan-family assays can be expected to be less prone to mutation induced errors, though routine comparison of primer target sites with observed viral strains is prudent. Further consideration must be given to the appropriate number of targets contained in a pan-family test to increase the likelihood that a novel pathogen is captured by an established assay, as well as the appropriate validation processes which should be undertaken following pathogen emergence before the approved use of a pan-family assay.

Our analysis has highlighted several limitations of reported pan-family tests resulting from an over-optimization on observed sequence variation rather than pre-emptive variation predicted from highly conserved protein motifs. Indeed, with the target regions applied by the MPC assay all possible silent mutations were observed, emphasizing the importance of including unobserved, but likely variation. Broadening of primer degeneracy with the strategies described in this work as well as routine surveillance studies of novel variation in key animal populations to detect unexpected genetic diversity may assist in ensuring ongoing effectiveness of pan-family assays. However, highly degenerate primers, such as the modified primers described in this work, are likely to have reduced test sensitivity to viral load. This may be partially counteracted through the use of universal base analogues such as inosine or 5-nitroindole[32] to reduced degeneracy or avoided through more judicious targeting of alternative highly conserved genomic motifs[6]. An alternative strategy may be the use of more relaxed reaction conditions to accommodate minor primer mismatches, though this may further reduce assay specificity[28]. These limitations must be appropriately characterized as part of pan-family assay development if to be applied in a clinical context.

A panel of pan-family assays would be required to provide a more robust tool for combating novel pathogens, with priority given to viral families associated with previous epidemics. Pan-family assays have been described for multiple viral families associated with recent epidemics, including influenza A viruses (Swine Flu)[33], filoviruses (Ebola Virus)[34] and flaviviruses (Zika Virus, Dengue Virus, Yellow Fever Virus)[35], and a similar analysis to the one presented here should be performed to assess the specificity and sensitivity of these assays for novel pathogens within the context of common viral species. Consistency in reaction conditions between pan-family assays or multiplexing of several assays into a single kit may further simplify the application of these tools during emerging pandemics.

A cost-effectiveness analysis of stockpiling pan-family assays is beyond the scope of this work given the novelty of the proposed testing strategies, and the unpredictability of pandemic emergence and characteristics. Most components required to run pan-family assays (nucleic acid-extraction kits, enzymes and buffers) may already be sufficiently stocked by diagnostic laboratories or may be able to be repurposed for routine testing or research applications towards the end of their shelf life. The only unique components required are the specific test primers, which depending on oligonucleotide modifications, number of reactions and production volume can be expected to cost below 0.5USD/test, with a shelf life of at least 2 years. However, to increase the simplicity and rapidity of testing it may be practical to stockpile all reagents required to run a pan-family assay as part of a self-contained test-kit, despite the additional cost. Additionally, due to the less restricted targets of pan-family assays, they may be applied more routinely during local epidemics of common pathogens. These assays may be preferable in low resource environment where maintenance of diverse species-specific testing is not possible.

Strategic stockpiling of pan-family viral assays is a proactive alternative to current viral disease test strategies which may expedite testing during emerging pandemics. By bridging the period in which species-specific test are in short supply these assays have the potential to greatly assist management of emerging public health emergencies through prioritization of high-resolution testing or isolation measures, despite limitations in test specificity due to cross-reactivity with common pathogens. Extensive further development, validation and certification of pan-family assays is needed prior to application in broad clinical contexts. With appropriate design these tools may allow informative and high-throughput screening of millions of individuals within days of pathogen emergence.

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Conflict of Interest

Potential conflicts of interest. E.S. is a member of the MX3 Diagnostics board of directors and has partial ownership of the company. M.E and GC are employees of MX3 Diagnostics. E.S., G.C. and M.E are authors of patents related to diagnostic testing. E.S., G.C., M.E. report patent 63/026,971 pending for a method and system for population screening of pathogens.

No conflict. D.Z. and B.G

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Tables

Table 1. Frequency of common HCoV infection in highlighted populations

	Study Population	HCoV infection rate
Van der Zalm (2009)	230 respiratory samples routinely collected from 19 children across 6 months	**Overall – 8.7%** Asymptomatic – 7.7% Symptomatic – 9.1%
Gaunt (2010)	11,661 respiratory samples from 7,383 patients as part of routine respiratory virus screening.	**Overall – 2.3%** Asymptomatic - 1.08% 7-12 months old – 4.86%
Prill (2012)	1481 hospitalized children with ACI or fever and 742 controls.	**Overall – 7.5%** Hospitalized – 7.6% Asymptomatic – 7.1%
Cabeca (2013)	1087 respiratory samples from patients with ARI. 50 Asymptomatic controls.	**Overall – 7.7%** Asymptomatic Adults – 0.0% All ARI patients - 8.1% Patients with Comorbidities – 12.4%
Lepiller (2013)	6,014 respiratory samples collected for routine viral diagnostics.	**Overall – 5.9%** Immunosuppressed Patients– 6.7% During Local Epidemic – ~16%
Matoba (2015)	4,342 respiratory samples from pediatric patients with ARTI	**Overall – 7.6%** During Local Epidemic – 28.5%
Zhang (2015)	Meta-analysis of 489,651 patients with ARTI	**Overall- 2.6%**
Yip (2016)	8275 respiratory samples from patents with ARTI	**Overall – 0.9%** 0-10 years old – 0.9% Over 80 years old - 1.5%
Liu (2017)	3.298 respiratory samples from pediatric patients with ARI	**Overall – 2.4%** During Local Epidemic – 10%
Zeng (2017)	11,399 respiratory samples from hospitalized pediatric patients with ARTI	**Overall – 4.3%** 7-12 months old – 5.9%
Killerby (2018)	854,575 HCoV tests from 117 Laboratories as part of routine respiratory virus screening.	**Overall – 4.6%** During Local Epidemic – 12.4%
Heimdal (2019)	3,458 samples from hospitalized pediatric patients with ARTI and 373 samples from controls.	**Overall – 9.1%** Asymptomatic controls – 10.2% Hospitalized – 9.1%

ARI – Acute Respiratory Illness. **ARTI** – Acute Respiratory Tract Infection

Figure Legends

Figure 1

Alignment of 4 Reported Pan-Coronavirus Primer Targets and the SARS-CoV-2 Genome. IUPAC nucleotide code used where degenerate primers are reported. Each of the 5 rows shows a different region of the SARS2 genome. Primers targets with a 100% match to the SARS-CoV-2 genome highlighted with a gray box. Primers with mismatches between target sites and SARS-CoV-2 genome are not highlighted, with mismatched underlined/

Figure 1