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Development of Dual Genotyping Protocol for Agricultural Norovirus

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2021

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Norovirus is the number one foodborne pathogen globally and is commonly spread through fresh produce. In order to trace back an outbreak, public health officials have to genetically match the strain found in patient stool to a strain found on a food item. Identification involves obtaining the partial sequence of the VP1 gene for the genotype and the RdRp gene for the P-type. Together they comprise the dual genotype of a norovirus strain. The method for dual genotyping stool samples has recently been streamlined such that the VP1 and RdRp sequences can be amplified by a single RT-PCR reaction. Ideally outbreak investigations use uniform methods to identify outbreaks strains across sample types, but food matrices are much more variable in their composition. This represents a gap in methodology that is important to fill given the increasing genetic variability in circulating norovirus strains. Agricultural spaces are important sites of norovirus contamination on produce. I used irrigation water, worker hand rinses, and produce (melon, jalapeño, and tomato) rinses to adapt the single-step stool sample RT-PCR method for use with agricultural samples. In order to overcome issues in low viral load and inhibitory molecules, I went through many rounds of PCR condition optimization, including nested PCR, thermocycler conditions, and reaction solution components. Although using a nested PCR approach showed significant improvement in amplification, it was ultimately shown that the novel primer pair used with stool samples was not fit for widespread use with agricultural samples. While there are lessons learned through the protocol adaptation, these results indicate that alternative methods should be explored for dual genotyping norovirus in agricultural matrices.

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Introduction

Microbial Food Quality

Foodborne illness impacts about 48 million people in the United States (U.S.) every year (CDC, 2018). The top five pathogens spread through food are norovirus, *Salmonella*, *Clostridium perfringens*, *Campylobacter*, and *Staphylococcus aureus* (CDC, 2018). These pathogens induce many of the same symptoms including nausea, vomiting, and diarrhea (CDC, 2021). The severity of symptoms can vary from moderate to serious and life-threatening for all of these pathogens, although most patients are able to recover without medical intervention (CDC, 2022a). Leafy greens, vine-grown vegetables, and fruits are among the top five causes of foodborne illness, hospitalization, and death in the U.S. (Painter et al., 2013). Norovirus causes over half of all outbreaks spread by these raw produce items (Bennett et al., 2018).

Fresh food can be contaminated by microbes at almost any point throughout the supply chain. This includes contact with contaminated irrigation water or soil, harvesting and processing equipment, and infected agricultural worker hands whether they be the harvester, preparer, or anyone in between (Machado-Moreira et al., 2019). Given these various contamination points, the burden of foodborne pathogens will only continue to grow as produce consumption increases globally (Machado-Moreira et al., 2019). Additionally, the opportunity for viral contamination of agricultural goods increases when produce is imported internationally. This is of particular concern in the U.S. because of our reliance on imported produce. Every year, the U.S. imports $15 billion worth of fruits and vegetables from Mexico alone, making them our top international agricultural goods supplier (*Mexico*, n.d.). Extra processing steps and differing produce safety standards complicate the food supply chain, providing more time and space for contamination.

Infected food handlers throughout this supply chain are particularly consequential in the spread of norovirus on produce (Bennett et al., 2018).

To control the spread of disease through produce, the U.S. enacted the Produce Safety rule in 2015 under the Food Safety Modernization Act. This Rule regulates microbial quality, encompassing all types of food-borne pathogens, through testing of agricultural water using *Escherichia coli* as the primary indicator organism and treatment of biological soil amendments to reduce microbial load (FDA, 2022). *E. coli*, along with *Enterococci* and total fecal coliforms, are commonly used indicators of bacterial and viral contamination because concentrations of many other pathogens are often below laboratory detection limits (Ailes et al., 2008). The Produce Rule, among other safety regulations, also require facility level worker hygiene and equipment sanitation precautions (FDA, 2022). Beyond this, produce quality and safety are determined by visual checks for imperfections before sale (*Cantaloup Grades and Standards*, n.d.). These broad-stroke control measures appear to leave ample margin for contamination along the farm-to-table pipeline.

Norovirus

Norovirus on Produce

Norovirus causes over half of all foodborne illness in the U.S. annually, with fresh produce being a major vehicle of infection (CDC, 2021). Foregone income and healthcare costs related to norovirus illness amount to $2 billion in annual losses (CDC, 2021). There are many factors both intrinsic to the virus itself and related to the vehicle of contamination which may influence this prevalence. First, norovirus is highly infectious, with an ID_{50} estimated at 18 viral particles, and can be spread from an infected to susceptible person through fomites or

aerosolized vomitus (Teunis et al., 2008). For reference, the ID_{50} of SARS-CoV-2 has been estimated as ~500 viral particles (Prentiss et al., 2022). Secondly, the biological matrices within which the virus is shed (e.g., stool and vomitus) provide physical buffers against environmental threats to infectivity. Moreover, the proteins which comprise the viral capsid make it resilient to aerosolization and the low pH within the human stomach (Kotwal & Cannon, 2014). These characteristics allows norovirus to persist in various environmental matrices for weeks or even months and remain resistant to many techniques that are typically used to reduce bacterial contamination on food such as freezing or heated processing (Machado-Moreira et al., 2019). Furthermore, norovirus is often associated with produce items that are eaten raw with no processing step to eliminate or reduce the viral load, contributing to the significance of these vehicles in the continuing burden of disease (Sobolik et al., 2021). As such, both the nature of the vehicle of contamination and norovirus' intrinsic characteristics lend themselves to maintaining high viral infectivity.

Genome and Recombinants

Norovirus is a non-enveloped single-strand RNA virus with a genome of about 7.5 kilobases (kb) containing 3 open reading frames (ORFs) (Chhabra et al., 2019). ORF1 encodes 6 non-structural proteins, including the RNA-dependent RNA polymerase (RdRp) at the 3' end of the reading frame. ORF2 encodes the major structural capsid protein VP1 with shell and protruding domains while ORF3 encodes the minor structural capsid protein VP2 which comprises the binding domain (Chhabra et al., 2019). Noroviruses are classified primarily through their overarching genogroup, determined by VP1 gene sequence diversity and enumerated GI through GX, with GI and GII being the dominant strains impacting humans.

Strains are further classified into one of 49 different genotypes based on the complete sequence of their VP1 gene and 60 P-types based on the partial sequence of the RdRp gene (Chhabra et al., 2019). For many years, norovirus identification only sequenced the RdRp gene, before surveillance methods shifted to the VP1 sequence for determination of the genogroup and genotype (Chhabra et al., 2019). Recent epidemiological trends have emphasized the importance of obtaining both the genotype and P-type.

Emergent outbreak strains tend to be recombinants, meaning they contain a novel combination of genotype and P-type (Cannon et al., 2017). Recombination events occur when an individual is coinfected with two (or more) norovirus strains; the viruses enter the same cell and, during viral replication, polymerases disassociate from their original template and switch strands mid-transcription (Ludwig-Begall et al., 2018). The norovirus genome contains an internal promoter sequence at the ORF1/ORF2 junction, which can stall the polymerase and allow it to template switch (Bull et al., 2005). The resulting transcript has a different combination of RdRp and VP1 genes than either of the parent strands. Norovirus outbreaks frequently involve two or more strains, especially when they originate from an environmental reservoirs or infect people with compromised immune systems (Ludwig-Begall et al., 2018). Given the ability of norovirus to persist in the environment, reservoirs are able to accumulate strains from multiple sources over time. Infected immunocompromised individuals exhibit a lack of immune pressure and extended periods of viral shedding which provide opportunity for co-infection as well as recombination events (de Graaf et al., 2016).

Norovirus recombinants pose unique challenges for public health officials attempting to monitor and mitigate transmission. The variances of the capsid protein, particularly the VP1 gene, are said to affect the infectivity of a strain. Different capsid protein genotypes can impact

virus-host interactions and/or stability in the environment such that the new strain can escape existing herd immunity to other predominant genotypes and then take hold in the population (Tohma et al., 2021). Different RdRp types may have different mutation rates which itself could give rise to a new strain. These different polymerases could also have varying transcription rates which impact the amount of viral particles an infected individual sheds (Tohma et al., 2021). Additionally, emerging recombinants create a moving target for vaccine development, as a viable vaccine would likely require capsid-specific epitopes (Ludwig-Begall et al., 2018; Rackoff et al., 2013). Thus, understanding the genetic diversity of outbreaks is important to understanding molecular mechanisms of strain emergence and to generating an effective vaccine.

Outbreaks and Transmission Patterns

Outbreaks of norovirus in the U.S. tend to peak in the wintertime (from October through April) although transmission is sustained throughout the year. These seasonal outbreaks may be driven by reduced antiviral vitamin D production, closer contact among susceptible people as they remain indoors, and ecological factors (Rohayem, 2009). Outbreaks have been positively associated with low temperature, low humidity and high rainfall, although these findings differ by region (Shamkhali Chenar & Deng, 2017). Off-season transmission may be sustained by asymptomatic transmission (Sobolik et al., unpublished data). This off-season transmission may provide opportunity for coinfection and recombination events within individuals.

Impact of Climate on Disease Trends

Climate is defined as the average weather for a region over a 30-year time period, whereas weather is the short-term (on the scale of minutes to months) variations in the

atmosphere including temperature, precipitation, cloud cover, wind, and atmospheric pressure (NASA, 2017). Above, the impacts of seasonal variations in weather were described as they related to norovirus transmission patterns. Seasonal variations depend on the region but tend to repeat the same patterns from year-to-year. Regional climate predictions allow for individuals, public agencies, and industries to prepare for regular seasonal variations.

Climate change has the potential to reshape established transmission patterns. Climate change is expected to increase global temperatures, exacerbate sea-ice melting, shift climate zones poleward, and increase the frequency and intensity of extreme weather events like heavy precipitation, heatwaves, and drought (Cissé et al., 2022). These impacts have the potential to shift norovirus' (and other pathogens') transmission patterns through ecological effects on reservoirs and changes in host dynamics (Rohayem, 2009). For example, flooding of agricultural fields can directly contaminate produce before consumption. Flooding can also bring norovirus in contact with susceptible populations directly by overwhelming and compromising wastewater infrastructure (Cissé et al., 2022). Moreover, flooding events can create a pool of different strains which can cause an outbreak in tandem and create opportunity for recombination events. Additionally, humans will have to adapt to climate change in ways that have the potential to worsen outbreaks. Agricultural practices already differ region by region and, as climate zones shift, these practices will change and create new challenges for food safety (McMichael et al., 2012). Climate change-driven weather events has already begun to ravage some regions to the point of displacing the people who live there (Levy & Patz, 2015). This forced migration can introduce pathogenic strains, including norovirus, to new populations and create densely populated environments for them to spread (Cissé et al., 2022). The variability of these climate-

related effects makes them extremely hard to prepare for and will make monitoring of outbreak trends all the more important.

Identification Challenges

Outbreak Investigations

Investigation of norovirus outbreaks follow the same steps as other foodborne illness inquiries (CDC, 2022b). Typically, as an enteric outbreak occurs, some of the most severely symptomatic individuals seek treatment and their doctor will order collection of a clinical sample to identify the pathogen. If the sample is found to contain an enteric pathogen, the hospital alerts a local health department who collects the stool specimens for genetic sequencing. Public health officials are continuously tracking data from local health departments about when and where people become sick with the same pathogens or pathogens with similar symptoms. Once an outbreak is identified, the public health investigators may contact the patient for a hypothesis-generating interview or shotgun questionnaire. These inquiries help establish a list of food items and other exposures that the patient may have had leading up to their illness. The food exposures which cases have in common help investigators narrow down the possible sources of infection. From there, epidemiological case-control studies and laboratory testing of potential food sources allow investigators to trace the common food item throughout its production chain and determine at which step it became infected with norovirus, or whichever foodborne outbreak they are investigating. Their final step is to recall the food item, disinfect the food facility, or enact another control measure that will prevent further infections.

Currently, norovirus monitoring efforts in the U.S. are coordinated primarily through Centers for Disease Control and Prevention's (CDC) CaliciNet and NoroSTAT systems (CDC,

2022c). CaliciNet is a surveillance network of federal, state, and local public health laboratories that submit electronic genetic sequence and epidemiological data after a norovirus outbreak. Data is only admitted to the database if it is collected and analyzed by CaliciNet-certified laboratories. There are 34 CaliciNet labs in 29 states across the U.S. and non-certified labs may participate by sending specimens to one of five CaliciNet Outbreak Support Centers. NoroSTAT is a network of 12 state health agencies and CDC that utilize standardized reporting practices to improve timeliness and completeness of the CaliciNet database. NoroSTAT and CaliciNet feed into the more general National Outbreak Reporting System (NORS) which tracks outbreak reporting for all enteric diseases. Notably, this surveillance network relies on outbreak samples emerging and then works backwards to identify sources and control the spread. It may be weeks after a patient develops symptoms that they are contacted for an interview or questionnaire, and weeks still until the food source is identified and laboratory confirmed.

Methodological Considerations

Because it is not yet viable to culture norovirus *in vivo* (Tan, 2021), testing of norovirus outbreak samples typically involves a form of reverse transcription polymerase chain reaction (RT-PCR) to transcribe a section of the RNA genome into DNA and then amplify the DNA for sequencing (Chin et al., 2022). If the thermocycler being used has capacity for quantitative PCR (qPCR), then the cycle threshold (Ct) value can be used as a measure of initial viral load, with low Ct indicating more virus. If the thermocycler performs conventional PCR, the amplicons are electrophoresed on an agarose gel and visual confirmation of the band of correct size will indicate successful amplification. From there, the PCR product can be purified and sequenced.

As of the 1990's, RT-PCR with subsequent gel analysis and sequencing has been the standard method of detection for noroviruses set by the CDC (Moe et al., 1994). This initial method distinguished sample sequences with the amplicons from a primer pair that targeted the RdRp region of the genome. Given the increasingly evident circulation of recombinants, modern methods often involve primers that cover the RdRp region of ORF1 and a second reaction using primers that cover the VP1 region of ORF2 for dual genotyping (van Beek et al., 2018). Identification of recombinants through sequencing is crucial for monitoring global outbreak trends and aiding in the search for a viable vaccine design.

Only recently has the method for identifying recombinants in stool samples been streamlined. The CDC developed a RT-PCR protocol for dual genotyping GI and GII noroviruses using a single primer pair (Chhabra et al., 2020). They did so by using a forward primer that attaches at the 3' end of ORF1 and a reverse primer that attaches at the 5' end of ORF2. As such, this PCR amplifies part of the RdRp gene and part of VP1 gene as a single roughly 580 base-pair amplicon that is broad enough to determine both the P-type and genotype.

This protocol development advances norovirus monitoring for many reasons. First, the authors were able to identify 90% of their 2,663 total known-positive stool samples (Chhabra et al., 2020). Moreover, these 2,392 successfully dual genotyped samples represented all but one of the known circulating genotypes and P-types. This shows that the technique could be applied to a broad range of samples and get consistent detection for nearly all of them. Another strength of this method, as shown by the authors, is that the limit of detection for the GI and GII primers was as low as 5 viral particles per reaction and 50 viral particles per reaction, respectively. This broad range of applicability and low limit of detection are hallmarks of robust primer design among PCR methods.

Difficulties with Food Matrices

Testing stool samples, however, is only one step in the process of controlling a food-borne outbreak. In order to enact control measures, public health investigators need to similarly identify norovirus on a food product. Identification in food matrices uses the same microbiological techniques but carries additional challenges that stool does not. With stool samples, the sample is prepared for RT-PCR by being suspended in solvent and then RNA is extracted using a commercially available kit (Chin et al., 2022). Often, patients are shedding virus in stool in large enough quantities (on the order of 10^8 viral copies/g of fecal specimen (Chan et al., 2006)) so that enrichment or concentration of samples is not necessary (Chin et al., 2022). This is not the case in agricultural sampling. Despite its strong environmental persistence, norovirus is often found in low concentrations on produce and in water (Van Pelt et al., 2018). Additional difficulties arise from PCR inhibitors that are commonly found within agricultural matrices (Elizaquível et al., 2014). These can lead to false negative results (i.e., an artificially high Ct value or lack of a detectable band in a gel). Overcoming the low viral load and inhibitory factors are difficulties that limit agricultural norovirus detection techniques from being identical to those used in stool samples. As such, the standard method for identifying recombinants in agricultural matrices still involves two separate PCR techniques for determining the genotype and P-type (Kageyama et al., 2003; Shaheen et al., 2019).

Clean Greens

The Clean Greens team, led by Dr. Leon at Rollins School of Public Health, has been working to advance methods used to quantify infectious norovirus on various fresh produce

commodities and matched environmental samples since 2010. To this end, they have collected produce rinse samples from three different commodities with matched irrigation water and worker hand rinse samples, all of which have been genotyped using a hemi-nested RT-PCR approach and tested for inhibition using RT-qPCR (Bartz et al., 2016). These samples are useful for validating a new technique for sequence amplification because they provides sample-specific information on characteristics that will impact the success of a new assay. Additionally, the new sequences can be matched to track the infection source on the farm, further validating the method for its intended use.

Purpose

The standard method for agricultural detection of norovirus needs to be streamlined to keep up with advancements in stool detection methods so that outbreak investigations and control measures can be implemented rapidly and with uniform methodology. Much like the new single-step stool protocol, an ideal method for agricultural matrices would have high specificity and be able to consistently and definitively identify a range of genotypes and P-types. Below are the specific aims to achieve this goal.

1. Ensure the single-step protocol produces consistent results with our positive control inoculum and outbreak stool samples.
2. Apply the stool sample protocol to RNA extractions from collected irrigation water, worker hand rinses, and produce rinse agricultural samples.
3. Adjust the protocol to address challenges in RNA inhibition and low viral load within the samples.

Significance

If successful, this protocol could be implemented immediately by all public health laboratories which currently perform norovirus identification. It utilizes standard PCR techniques and simple primer design. Although different produce commodities present different amplification challenges, the various sample types and adaptation techniques used here would support its application in different laboratory settings. This would simplify outbreak investigations by aligning the primers used for both patient stool sample testing and food product testing.

Simplified and ubiquitous outbreak monitoring is going to be increasingly necessary as climate change continues to impact disease dynamics. As discussed above, the seasonal variability in transmission patterns, which are already only loosely understood, will become increasingly unpredictable. The food system's transmission precautions, which are already imperfect, will be subject to change as climate zones shift the growing seasons and viable produce commodities. The uncertain transmission patterns and food safety efficacy will necessitate diligence on the part of public health professionals monitoring norovirus among other food and water borne diseases. This laboratory technique would aid in streamlining these monitoring processes.

Methods

Sample Sources

Stool Specimens

Positive control inoculum samples of Norwalk (GI.1[P1]) and Snow Mountain (GII.2[P30]) came from norovirus challenge studies conducted by Dr. Christine Moe at Rollins School of Public Health (Leon et al., 2011; Lindesmith et al., 2005). The positive control stool samples used for confirmation of the protocol came from student patients of a food-borne norovirus outbreak at Georgia University of Technology (GT) in 2017 (Leon et al., unpublished data). Each of the 4 samples used in this project were voluntarily collected from different student patients of the GT outbreak on different days. Sample collection, de-identification, stool re-suspension, and RNA extraction was overseen by the lab groups of Dr. Juan Leon and Dr. Christine Moe within the Hubert Department of Global Health at Rollins School of Public Health. Outbreak samples were also previously confirmed to contain GI or GII norovirus by quantitative RT-PCR (RT-qPCR) of the highly conserved region between Open Reading Frames 1 and 2 (ORF1 and ORF2) of the genome. (Leon et al., unpublished data). **Table 1** shows the previously determined genotype of each sample used.

Table 1. Inoculum and Stool Sample Known Characteristics

Sample ID	Sample Source	Genotype
Norwalk Virus (NV)	Challenge study inoculum	GI.1
Snow Mountain Virus (SMV)	Challenge study inoculum	GII.2
295	Outbreak study	GI.3
594	Outbreak study	Unknown*
298	Outbreak study	GII.2
180	Outbreak study	Unknown*

* Samples with unknown genotype were unsuccessful in the sequencing protocol.

Agricultural Specimens

The agricultural samples used for the adaptation of this protocol come from the ongoing Clean Greens research conducted by Dr. Leon at Rollins School of Public Health, and colleagues at North Carolina State University and Universidad Autónoma de Nuevo León. Sampling took place on 11 cantaloupe melon, jalapeño pepper, and tomato farms across the Mexican states of Nuevo León and Coahuila from May 2011 to December 2012 as described previously (Bartz et al., 2016). This location was chosen for its production of target produce and large export volume to the U.S. All farms used drip irrigation and well water sources. Three had packing facilities on site. Four types of agricultural samples were used in this project: produce rinses, hand rinses, source water, and irrigation water. Produce rinse samples were taken both pre- and post-harvest, and matched to worker hand rinses after harvesting. Well water was sampled from the source pump and irrigation water were sampled from hoses in the field; both water samples were matched to produce and worker rinse samples pre-harvest (Bartz et al., 2016).

All agricultural samples were previously confirmed norovirus positive (GI or GII) by RT-qPCR in the Leon Lab at Rollins School Public Health. Cycle threshold (Ct) output values from the RT-qPCR were used as indication of the viral load in each sample, with low Ct correlating to more viral particles. Inhibitors present in extracted RNA samples were calculated through the amplification of competitive Internal Amplification Controls (IAC) according to ISO protocols (Prince-Guerra et al., unpublished data). If IACs were not detected, the sample was identified to contain inhibitor molecules. Ethical approval for the study was overseen by Emory University and Universidad Autónoma de Nuevo León (IRB 00035460) and all samples were de-identified before my use. **Table 2** shows data known about each sample prior to the start of the assays.

Table 2. Agricultural Sample Known Characteristics

Sample ID	Sample Type	Produce Commodity	Ct Value	Inhibition	Genotype
110	Source Water	Tomato	29.32	yes	GII.2
507	Produce Rinse	Melon	30.17	yes	GII*
107	Irrigation Water	Tomato	29.02	no	GII.2
168	Produce Rinse	Jalapeño	33.63	no	GII*
144	Produce Rinse	Tomato	35.69	yes	GII.6
624	Hand Rinse	Jalapeño	35.45	yes	GII*
424	Produce Rinse	Melon	35.46	no	GII.6
70	Produce Rinse	Tomato	35.67	no	GII*
493	Hand Rinse	Tomato	39.36	yes	GII*
134	Produce Rinse	Tomato	38.91	yes	GII*
86	Produce Rinse	Tomato	39.58	no	GII*
403	Irrigation Water	Melon	38.87	no	GII*
421	Hand Rinse	Melon	29.42	yes	GII.2
328	Irrigation Water	Melon	30.33	yes	GII.2
293	Irrigation Water	Melon	30.77	no	GII.2/GI.1**
413	Irrigation Water	Melon	31.34	yes	GII.2
43	Produce Rinse	Tomato	37.51	no	GI*
45	Produce Rinse	Tomato	36.19	yes	GII*
68	Produce Rinse	Tomato	31.74	no	GII.6
75	Hand Rinse	Tomato	34.83	yes	GII*
76	Produce Rinse	Tomato	34.10	yes	GII*
428	Produce Rinse	Melon	31.64	no	GII.2
374	Irrigation Water	Melon	32.03	yes	GII.2
87	Irrigation Water	Tomato	32.1	no	GII.2
254	Produce Rinse	Jalapeño	32.30	no	GII.2
240	Source Water	Jalapeño	33.20	yes	GII.2
78	Produce Rinse	Tomato	38.12	no	GII*
85	Hand Rinse	Tomato	35.77	no	GII.2
87	Irrigation Water	Tomato	32.1	no	GII.2
90	Source Water	Tomato	32.28	no	GII*
95	Hand Rinse	Tomato	38.86	yes	GII*

*Samples were amplified by RT-qPCR (Ct < 45) but unsuccessful in the sequencing protocol.

**Sample contained both GI and GII and was tested in this project for both strains.

Amplification and Sequence Determination

Overview

My adaptation process consisted of many iterations, beginning with stool samples and proceeding with agricultural samples, but the iterations share their core components. For GI norovirus samples, previously identified by RT-qPCR, I used forward primer MON432 and reverse primer G1SKR. For GII samples, I used forward primer MON431 and reverse primer G2SKR. See **Table 3** for primer sequences, positioning, and PCR amplicon length. Each reaction was carried out using the same final reaction conditions: 5µL of 5X Qiagen RT-PCR Buffer Solution, 1µL of Qiagen OneStep RT-PCR Enzyme Mix, 1µL of 10mM dNTP mix, 0.5µL of 100 nmol forward primer, 0.5µL of 100 nmol reverse primer, 20 U RNase Inhibitor, 11µL RNase free water and 5µL of the sample for a total reaction volume of 25µL. Reactions which used Q-Solution (Qiagen, Germantown, Maryland) differed only in the use of 6µL RNase free water and the addition of 5µL of Q-Solution.

Table 3. PCR Primer Details

Genogroup	Primer Name	Sequence (5' - 3")	Mean Annealing Temperature	Genome Position	Amplicon Length
GI	MON432 (+)	TGG ACI CGY GGI CCY AAY CA	65.1℃	5093	579
	G1SKR (-)	CCA ACC CAR CCA TTR TAC A	52.4 ℃	5671	
GII	MON431 (+)	TGG ACI AGR GGI CCY AAY CA	61.4 ℃	4820	570
	G2SKR (-)	CCR CCN GCA TRH CCR TTR TAC AT	59.2℃	5389	

The basis of this process was the genotyping protocol as written by Preeti Chhabra and her colleagues at the CDC (Chhabra et al., 2020). Their PCR thermal profile was as follows: reverse transcription at 42℃ for 30 min, activation of *Taq* polymerase at 95℃ for 15 min, and 40 cycles of denaturation at 95℃, annealing at 50℃, and extension at 72℃ for 1 min each, followed by a final extension for 10 min at 72℃ and cooling down to 4℃. As described below, only the reverse transcription and annealing temperatures were altered throughout my iterations.

Following PCR, samples (including positive and negative controls) were mixed with EZ-Vision DNA Dye (VWR Life Sciences, Atlanta, Georgia) and electrophoresed on a 2% agarose gel at 100 V for 1 hour, alongside a 100 kb DNA ladder. Visualization was achieved with the ChemiDoc XRS+ Molecular Imager and Image Lab Software (BioRad, Hercules, California). Positive samples were confirmed by visual check of a strong band at 579 bp for GI and 570 bp for GII. Samples with a positive band were purified, either using the Qiagen Gel Purification Kit or with the Qiagen Column Purification Kit and according to their manual (Qiagen, Germantown, Maryland). Premixed sequencing reactions were sent with forward and reverse primers to Azenta Life Sciences according to their submission guidelines (Azenta, South Plainfield, New Jersey).

With the raw sequence files, I analyzed the quality of the read-outs and generated a consensus sequence using Seqman Ultra software from DNAStar available through Emory University. The consensus sequences were entered into the Calicivirus Typing Tool (https://calicivirustypingtool.cdc.gov/) for a final dual genotype determination. The Typing Tool assigned a specific genotype and P-type if the entered sequence matched more than 80% of a

reference genotype or P-type. It also provided an overall "Percent Identity" based on an NCBI Blast search of the entire sequence.

Modifications Made During Stool Samples Confirmation

In order to establish that the protocol could work on our laboratory controls, the inoculum samples (NV and SMV) were run through a single RT-PCR with the exact thermal profile published by Chhabra et al. as described above.

To strengthen the amplification, I ran nested RT-PCR reactions of SMV and outbreak samples with the same thermal profile as written by Chhabra et al. First round RT-PCR products were diluted 1:5 and 1:10 with RNase-free water before being used as the template for a second PCR. The second PCR did not include a reverse transcription step but otherwise had the same thermal profile. The positive samples which were diluted 1:5 were extracted via gel purification and the 1:10 diluted samples were extracted via column purification as described above. Sequencing and analysis were completed as described above.

Modifications Made During Agricultural Adaptation

I continued with nested RT-PCR and a 1:10 sample dilution between PCR for the agricultural samples. The nested RT-PCR and dilutions were applied to SMV and 31 total agricultural samples representing a range of Ct values, inhibition, and hemi-nested sequencing results.

Two different approaches were taken to address incompatibility of annealing temperatures between the forward and reverse primers. Ideally, a primer pair would have annealing temperatures within 5°C of each other such that they anneal and fall off concurrently

throughout the reaction cycles. As shown in **Table 3**, this was not the case for the GI primers and all ideal annealing temperatures were above 50℃. To troubleshoot this, I ran samples on a 5℃ incremental annealing temperature gradient ranging from 50℃ to 65℃. Ultimately, the annealing temperature was optimized at 55℃. I also ran samples with the addition of Q-Solution. The manufacturer recommends the addition of this reagent in order to improve annealing efficiency of GC-rich primers and primers that have trouble annealing in general.

Additional protocol adjustments were made to deal with PCR inhibition and improve RT-step efficiency. To address inhibitory molecules present in some samples, I ran samples with multiple different pre-PCR dilutions ranging from 1:2 to 1:50, with 1:10 proving to be the most efficient dilution factor. To further increase the amount of PCR products, I optimized the reverse transcription temperature for the specific reverse transcriptase used in this project, increasing it from 42℃ to 50℃.

Results

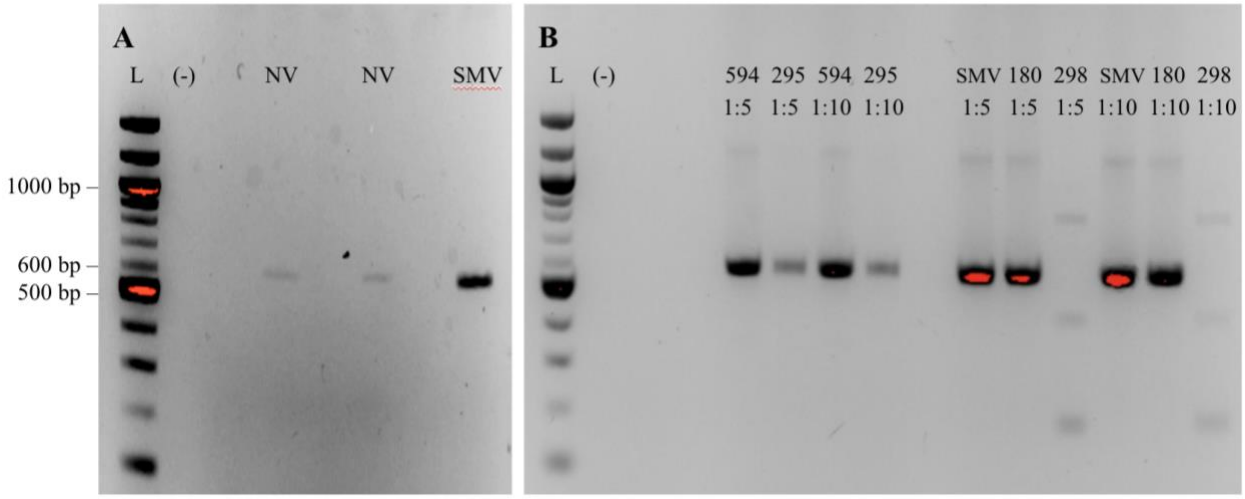

Figure 1. Gel visualization of norovirus stool samples amplification reveals improvements with a nested PCR. Both gels were run with a 100 kb ladder (L) in the first well and a negative PCR control (-) in the second lane. Bands appeared at the expected amplicon size of 579 bp for GI samples (NV, 594, 295) and 570 bp for GII samples (SMV, 180, 298). Panel A shows gel results for the single-step RT-PCR while Panel B shows gel results for the nested RT-PCR. Red marks indicate a particularly high abundance of PCR product. In B, the between-PCR dilution factor is indicated below the sample ID.

The goal of the first single-step RT-PCR run was to confirm that the dual genotyping protocol could successfully amplify standard inoculum strains for sequencing. To this end, I applied the protocol as written by Preeti Chhabra et al. (2020) on NV and SMV, our lab's standard positive control samples. The gel (**Figure 1A**) showed that NV produced a band of faint intensity while SMV produced a band of strong intensity, each at their expected amplicon size of 579 and 570 bp respectively. Although SMV amplicons seemed to be present in high concentration based on the band intensity, the weak presence of NV amplicons on the gel

indicated that it was unlikely that there would be enough NV product to sequence. Thus, these samples were not purified for sequencing. The bands in **Figure 1A** are exemplary of what will henceforth in this project be considered "weak" and "strong" respectively: a thin gray line likely does not contain enough product while a thicker, more opaque black line -comparable to the bands along the DNA ladder- was certainly sufficient to send off for sequencing. This trial revealed that the protocol, as written, does not generate unambiguously sufficient product of both the GI.1[P1] and GII.2[P30] strains to send for Sanger sequencing.

By introducing new strains and a nested-PCR approach, I hoped to address the failure of the first trial to amplify abundant product of multiple strains. Four new outbreak stool samples were run alongside SMV. Each sample was amplified in duplicate in an RT-PCR, then one duplicate was diluted 1:5 and the other diluted 1:10 with RNase-free water before being run in a second standard PCR. The gel (**Figure 1B**) showed consistently strong intensity bands for samples 594, SMV, and 180 and moderate band intensity for sample 295. Sample 298 showed bands at 3 non-target sizes, indicating non-specific amplification. All samples showed no visible difference in band intensity between the different dilution factors. These results suggest that the nested PCR approach can produce strong band intensity and consistent results for a variety of sample strains.

Table 4. Stool and Inoculum Sample Sequencing Summary

Sample	Genogroup. Genotype [P Type]	Dilution between PCRs	Average Quality Score	Consensus Sequence Length (nt)	Genotype Matching	P-Type Matching	NCBI Blast Percent Identity
594	GI.3[P13]	1:5	55	559	97.7%	92.4%	98.93%
		1:10	56	559	97.7%	92.4%	98.93%
295	GI.3[P13]	1:5	43	564	97.7%	79.0%*	98.58%**
		1:10	56	581	97.7%	92.4%	98.59%
SMV	GII.2[P30]	1:5	54	512	100.0%	99.4%	100.00%
		1:10	54	523	100.0%	100.0%	100.00%
180	GII.2[P16]	1:5	54	523	100.0%	98.8%	99.81%
		1:10	55	545	100.0%	98.8%	99.81%

* This matching percentage did not meet the Calicivirus Typing Tool's threshold, so a P-type was not called

**This Percent Identity is based on genotype only

To determine if the nested PCR products were able to be sequenced, I purified the products, sent them in duplicates for Sanger sequencing, analyzed the quality of the readouts, and created a consensus sequence using the Seqman Ultra software from DNAStar. The consensus sequences were entered into the Calicivirus Typing Tool which returned a Percent Matching score, relating the consensus sequence to reference sequences of each genotype and P-type, along with an overall Percent Identity from NCBI Blast (**Table 4**). The genotype and P-type were only returned if Percent Matching scores exceeded the threshold of 80%. For all but one of the samples, the consensus sequences showed very high Matching scores (> 90%) and

each dilution returned the same dual genotype result (**Table 4**). The 1:5 dilution of sample 295 contained low quality read-outs which ultimately impacted the ability of the Typing Tool to determine its P-type. The genotype of 295, however, did meet the Typing Tools threshold and was in agreement with the dual-genotype determination from the 1:10 dilution. These results suggest that the nested PCR approach can be carried out for a variety of stool sample strains with consistent genotyping results that met or exceeded the Typing Tool threshold for determination.

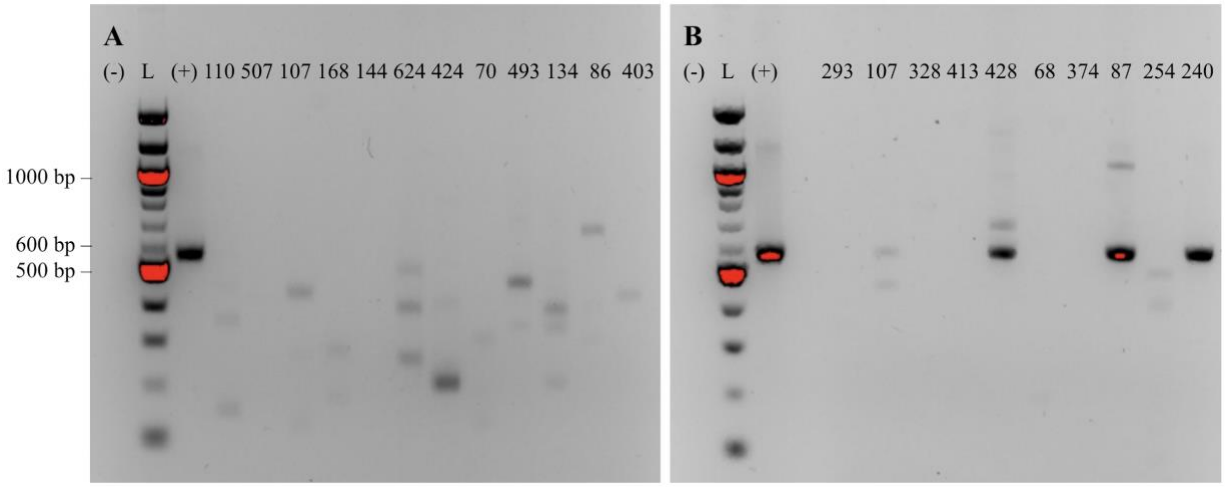

Figure 2. Gel visualization of agricultural samples run on nested PCR produced inconsistent results despite continual optimization of reaction conditions. Both gels were run with a negative PCR control (-) in the first lane, a 100 kb ladder (L) in the second lane and SMV positive PCR control (+) in the third lane. Numbered lane headers indicate the sample ID, referenced in **Table 2**. Red marks indicate a particularly high concentration at that fragment size. The expected band size was 570 bp. Panel A shows gel results from the first agricultural sample trial, with the same reaction conditions as the stool sample nested RT-PCR. Panel B shows gel results from the last of the agricultural sample trials, with a nested RT-PCR and optimized RT-

step temperature, annealing temperature, and pre-PCR dilution factor. Each trial was run with a different subset of samples listed in **Table 2**.

The many trials involving agricultural samples were adapted from one to the next with the goal of determining optimal conditions for consistent results with high sensitivity to a range of dual genotypes with low concentrations of viral RNA. To this end, I ran the nested RT-PCR with 1:10 dilution between rounds (**Figure 2A**), tested a range of annealing temperatures, tested a pre-PCR dilution for samples with inhibitors, tested the addition of Q-Solution to stabilize the primers within the reaction mixture, and ran an optimized RT step temperature (**Figure 2B**). **Figure 2** shows two gel results that are representative of the inconsistency in amplification of samples despite the optimization of these reaction conditions. The initial trial (**Figure 2A**), prior to any agricultural sample reaction optimization, resulted in no product of correct size and non-specific amplification in 8 of the 12 reactions. This indicated that further development of the protocol was necessary. The optimal conditions were as follows: an annealing temperature of 55℃, a 1:10 pre-PCR sample dilution for those containing PCR inhibitors, no added Q-solution, and a 50℃ RT step. The trials that tested and verified these reaction conditions (not shown here) resulted in only 2 bands of correct size and intensity. The final run (**Figure 2B**) was the result of many rounds of optimization for the conditions described above. It generated 4 bands of correct size: 3 samples (428, 87, 240) with strong band intensity and one sample (107) with moderately weak intensity. There was non-specific amplification in 4 of the 10 samples. The described inconsistency in specific amplification across the 31 total samples suggested that RT-PCR with these primers was not adequate for consistent amplification of agricultural norovirus samples.

Table 5. Agricultural Sample Sequencing Summary

Sample	Genogroup. Genotype [P Type]	Average Quality Score	Consensus Sequence Length (nt)	Genotype Matching	P-Type Matching	NCBI Blast Percent Identity
110	GII.2[P30]	55	540	100.0%	100.0%	100.0%
293	GII.2[P30]	56	540	99.6%	100.0%	99.6%
107	GII.2[P30]	56	537	100.0%	100.0%	100.00%
428	GII.2[P30]	24	512	91.2%	87.7%	91.99%
87	GII.2[P30]	54	539	100.0%	100.0%	99.81%
240	GII.2[P30]	56	530	100.0%	100.0%	100.00%

Despite the inconsistent amplification of our target with strong intensity within agricultural samples, it was yet to be determined if the few bands that did appear could provide dual genotype information. To this end, I purified the PCR products, sent them for Sanger sequencing, generated a consensus sequence, and analyzed the consensus sequence through the Calicivirus Typing Tool. All six of the samples which produced bands were able to be dual genotyped with high Matching scores and Percent Identities. Despite 107 showing the weakest band intensity on the agarose gel, only sample 428 had a low-quality score. This low-quality score did lower its Matching and Percent Identity but not below the Calicivirus Typing Tool's

threshold for determination. As such, although the protocol was inconsistent in its ability to produce specific and ample PCR products, the six successfully amplified samples were able to provide dual genotype information.

Discussion

Overview

The purpose of this project was to determine how the single-step RT-PCR protocol for dual genotyping norovirus from stool (Chhabra et al., 2020) could be adapted for use with agricultural rinse samples. The major adaptation step was using a nested PCR, which showed greater success among our positive control stool samples. Ultimately, however, consistent amplification among known norovirus-positive samples and strong intensity gel visualization was not feasible with these primers. Additionally, all agricultural samples which were successfully amplified were identified by dual genotyping as SMV, thus presenting no evidence that these primers are fit for a variety of sample strains. Therefore, despite the promise of the stool protocol, this approach presents too many challenges for it to be feasible for widespread use with agricultural samples.

The adaptation measure which showed the single most significant improvement in the protocol's ability to amplify known-positive samples was the adoption of a nested PCR approach. Nesting significantly increases sensitivity and yield of RT-PCR protocols (Goode et al., 2002). Traditionally, this approach utilizes two different sets of primers for the first RT-PCR and the subsequent PCR round. However, in an attempt to maintain the simplicity of Chhabra et al.'s work, this protocol used the same primer set for both PCR rounds. When using the same primers in nested PCR there is increased potential for primer-dimer hybridization. This occurs when the forward and reverse primers bind to each other and the Taq polymerase generates a small nonspecific product which can be carried on from one PCR to the next (Goode et al.,

2002). Although the gel doesn't show pervasive primer-dimers across all sample runs, non-specific amplification was much more pronounced in the nested PCR runs (**Figure 1**).

Despite the success of the nesting technique, PCR optimization can only go so far. I optimized many of the reaction elements which may prevent full amplification, and still found that consistent and strong intensity amplification across all samples was not feasible. Altering reaction components such as annealing temperature, RT temperature, and reaction solution makeup can help improve sensitivity and specificity (Cale et al., 1998) but primer design is crucial for successful RT-PCR (Li & Brownley, 2010). The primers used here bind to regions in the RdRp gene and VP1 gene that vary from strain to strain (Chhabra et al., 2020). It is inevitable that some strains will have genotypic differences to which the primers bind weakly and result in weak intensity or unsuccessful amplification. The primers' inability to sequence NV, which was confirmed in correspondences with Dr. Chhabra, is exemplary of this issue. The ambiguity codes used in the primer sequences (e.g., I, Y, R, and H) are meant to account for genotypic variation by having a mixture of primers with different bases at those sequence locations. This solution to genotypic variation appears to be imperfect for this application based on the amount of unsuccessful and non-specific amplification among these samples. Thus, despite optimization, this primer design cannot consistently detect a variety of samples from the agricultural context.

It is possible that the nature of environmental norovirus samples contributes to this issue of inconsistent and weak intensity amplification. The samples used in this project have an extremely low concentration of norovirus and many contain inhibitory molecules (**Table 2**). Sparsely concentrated samples may not contain enough starting material for all the primers and polymerase to bind and for the PCR to run at full efficiency. Low concentration is a known characteristic of environmental viral samples in general (Williams-Woods et al., 2022) and may

make any PCR assay impractical for successful wide-spread use on this sample type. Compounds like polysaccharides and humic, fulvic, or tannic acids are common in agricultural environments and are difficult to extract from samples. In PCR, these compounds can bind to the *Taq* polymerase and inactivate it, preventing product amplification (Kreader, 1995). The fact that Chhabra et al. were able to identify such a wide variety of norovirus strains (2020) but I was not able to do so with agricultural samples suggests that the sample type is a key issue.

All six agricultural samples which were dual genotyped were identified with high "Percent Identity" as our lab strain SMV. This is concerning because there is no evidence that SMV was a circulating strain during the time of sampling, or has been circulating at any time since then (Tohma et al., 2021). Rather, there is evidence of increasing sequence diversity among noroviruses. Thus, even if there were a SMV outbreak, it is highly unlikely that they would match the lab strain sequence with such a high percentage. It is also unlikely that this was due to the primers inability to pick up non-SMV strains, since the stool sample sequences showed a reasonable amount of genotypic diversity (**Table 4**). This suggests that there was contamination of samples with the SMV laboratory control. The most likely points of contamination were RNA extraction and PCR set-up because of the sequential use of multiple samples, lab controls and the same reagents. The dual genotyping results of these samples match the genotype identified previously by quantitative PCR, so it appears that contamination occurred before my project began.

Strengths and Limitations

Despite the ultimate failure of this approach for dual genotyping agricultural rinse samples, the adaptation methodology used here represents the strengths of this project. I

employed a variety of sample types that are representative of the sample types that may be used for source tracing in a foodborne outbreak (Williams-Woods et al., 2022). Additionally, I adhered to the original protocol as much as possible in order to maintain the integrity of the streamlined method (Chhabra et al., 2020). I used the exact same PCR kit, visualization method, and extraction kit as Chhabra. When nesting, I chose to only use their primer pair. The strength of the Chhabra et al. protocol was its simplicity and ease of implementation; the approach used here kept this elegance in mind when adapting for use with agricultural samples.

The methodology was not without limitations. First, there were other adaptation approaches not used here. For example, altering the cycle number or trying even more minute adjustments to annealing temperature could have possibly improved reaction results (Cale et al., 1998). The major reason additional avenues were not taken was the limited sample volume available for use with this project. Most sample volumes were around 20μL or less, so repeated trials of RT-PCR were not feasible. Another limitation was revealed in the evidence of sample contamination. If all the GII.2 agricultural samples are simply SMV contaminants then the primers were not actually used on a wide variety of genotypes and P-types, as was the original goal.

Next Steps

The findings here point to multiple avenues for further development of a streamlined norovirus detection and identification in agriculture method. This approach, if successful, would have been widely applicable because of the simplicity of the technique and ubiquitousness of the machinery (e.g., a thermocycler and gel electrophoresis manifold). Considerations regarding the ease of implementation should be important when focusing efforts on developing identification

techniques moving forward. Although nested PCR with two different primer pairs would be widely applicable and may reduce the presence of primer-dimers and non-specific amplification, it is not certain that it would improve the reaction sensitivity. The hallmark low viral concentration and inhibitory factors of environmental samples make any PCR approach uncertain.

Whole genome sequencing (WGS) as an alternative to PCR which has been successful thus far in identifying foodborne bacterial outbreaks. This technique uses advanced machinery and methodology to shear genomic material into fragments, sequence the fragments and then piece together the entire pathogenic genome (CDC, 2022d). WGS offers an even more streamlined approach than Chhabra's since the amplification and sequencing could be achieved with only one piece of equipment. Because this technology is new, however, it could only be implemented by laboratories which have the necessary machinery. The difficulties with agricultural samples due to low viral concentration and inhibition remain, but they may be more readily overcome. WGS can be approached non-specifically and tends to be more sensitive than PCR based methods (*Whole-Genome Sequencing (WGS)*, 2023). Nonetheless, it appears that pathogenic monitoring efforts are moving towards WGS, so perhaps it can become the standard in agricultural contexts as well.

Implications of Climate Change

Obtaining detailed genotypic information will be increasingly important as disease trends shift due to climate change. Seasonal weather patterns, which differ from region to region, have helped inform our understanding of norovirus transmission variability. For example, the wet season in North America which lasts roughly May through October (*Climate Change Knowledge*

Portal, 2021) tends to be followed by a period of norovirus outbreak peaks from October through April (Rohayem, 2009). It is possible that the mechanism behind this relationship is environmental norovirus transport through rainfall runoff, accumulation in various environmental reservoirs, and creation of transmission hotspots by the end of the season. Such a mechanism could create even more opportunity for co-infection and recombination events under the expected increase in extreme precipitation and climate zone shift. These two climate effects have the potential to create new environmental reservoirs and transmission hotspots. Monitoring efforts should strive for uniformity in techniques to improve the international collaboration that will be necessary for climate change mitigation and adaptation to infectious diseases.

Sample collection and genotyping methods will have to adapt to the changing environmental determinants of transmission. Increasingly extreme weather events will likely impact the makeup of environmental samples, including the viral and inhibitory molecule concentrations. Various biological components of soil may respond to climatic and biogeochemical factors differently, thus altering the overall microbiome dynamics (Jansson & Hofmockel, 2020). It is extremely difficult to predict whether concentrations of relevant components (virus and inhibitors) will increase or decrease, but methods of viral characterization in agricultural settings will be impacted by these changing conditions. To cope with this uncertainty, methods should be robust enough to accommodate worst case scenarios (low viral concentrations and increased inhibitor presence).

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