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Quantification and discovery of PCR inhibitors found in food matrices commonly associated with foodborne viruses

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ABSTRACT

Human norovirus is the leading cause of foodborne illness globally. Detection and quantification of norovirus commonly involves the use of reverse transcriptase quantitative polymerase chain reaction (RT-qPCR); however, the presence of inhibitory compounds in foods limit detection and accurate quantification. Although some studies have been done on PCR inhibitors from foods, many of them are over a decade old and do not investigate inhibition in contemporary one-step RT-qPCR-based detection chemistries. The purpose of this work was to quantify the degree of inhibition that occurs from inhibitory compounds found in produce (pectin) and mollusks (hemocyanin, glycogen)—foods commonly associated with norovirus outbreaks. RT-qPCR reactions containing different amounts of genomic bacteriophage MS2 RNA, a norovirus surrogate, were spiked with different concentrations of pectin (0.0625%–0.25% *w/V*), glycogen (1.25%–10%), and hemocyanin (0.0625%–0.25%). Past research has implicated glycogen as an inhibitory compound in oysters; however, even high levels of glycogen (10%) had no significant effect ($P > 0.05$) on amplification. Conversely, both pectin and hemocyanin caused complete inhibition at 0.25%, with no significant inhibition observed at 0.0625% ($P < 0.05$). Hemocyanin is abundant in the hemolymph of mollusks and previously untested as a PCR inhibitor. This work demonstrates that pectin and hemocyanin should be considered when testing produce and mollusk samples with PCR-based methods.

1. Introduction

Norovirus is considered the leading cause of foodborne illness in the United States and globally [1,2]. Human norovirus causes acute gastroenteritis, which may lead to severe vomiting, diarrhea and abdominal cramping. It has a low infectious dose, ranging from 18 to 2,800 genomic equivalents, and is resistant to many common active ingredients in sanitizers and disinfectants, along with traditional food preservation and processing methods [3,4]. This makes sensitive and accurate detection critical for control of noroviral transmission. Detection and quantification of norovirus involves the use of reverse transcriptase quantitative polymerase chain reaction (RT-qPCR); however, one challenge in its utilization is the presence of compounds in food that can inhibit detection and accurate quantification. Traditionally, nucleic acid extraction is used to avoid these substances, however, coextraction and purification of unwanted compounds is common [5].

Foodborne norovirus outbreaks occur year-round. The top two most implicated food categories in foodborne norovirus transmission are bivalve mollusks and produce, mainly being leafy greens and berries [6–8]. Berries, and particularly citrus fruits, contain higher levels of pectin; for instance, raspberries are around 0.97% pectin [9]. Pectinase treatment is sometimes implemented as a pretreatment in the detection of viruses in berries [10,11]. Demeke et al. [12] reported that the addition of apple pectin at 500:1 (polysaccharide to DNA) or 6% [*m/V*] pectin per reaction, did not display any inhibitory effect on PCR [12]. In contrast, a subsequent report by Pandey et al. [13] found that when using random amplification of polymorphic DNA (RAPD), 1,500 ng of pectin to 1.5 ng of DNA, or 12% [*m/V*] pectin per reaction, was needed to observe an inhibitory effect [13]. Other work has found inhibition of traditional PCR with as low as 0.5% [*m/V*] pectin [14]. One step RT-

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qPCR was not used in these studies; thus, none of these have been able to sensitively quantify the effect of pectin on TaqMan-based one step RT-qPCR quantification. In addition to being generally more sensitive than traditional PCR, one step RT-qPCR has an additional enzyme (reverse transcriptase) that can potentially factor into detection inhibition.

Previous reports suggest that the glycogen content of oysters is around 4.6% to 17.1%, depending on age and season of harvest [15]. Several early reports suggest that glycogen may be a PCR inhibitor [16,17]. When adding dilutions of oyster glycogen to PCR reactions containing poliovirus RNA, one study found glycogen at < 3.13% [*m/V*] did not inhibit amplification but higher concentrations did partially (6.25% glycogen; decreasing band intensity) or completely (12.5% and 25% glycogen; no visible band) inhibit PCR in older traditional PCR chemistries. Numerous other reports observe unknown inhibitors present in oyster extracts [18,19]. For example, Andersen et al. [20] observed inhibition when serial dilutions of oyster meal were added to PCR reaction mixtures [20]. This suggested that compounds other than glycogen could be the main inhibitory compounds present in oyster samples. Furthermore, the previously tested concentration of glycogen, 3.13% (*m/V*), is an unlikely amount to get co-extracted with RNA and end up in the final reaction. Kaufman et al. [21] investigated if mantle fluid within oysters could be used to detect norovirus rather than meat and found that the fluid significantly raised the detection limit in a PCR-based assay [22]. Although not explored further, the authors speculated that the oyster hemolymph could have been the source of inhibition observed. Hemocyanin is a blue respiratory protein found in mollusks' hemolymph that carries oxygen in a similar fashion to hemoglobin to mammals [23]. Hemocyanin is composed of several protein subunits, with each containing two copper atoms that together bind to a single oxygen. The hemocyanin structure differs between arthropods and mollusks, with mollusks having larger subunits. Hemoglobin has been reported to be a common PCR inhibitor found in blood, and hemocyanin mainly differs from hemoglobin by having a copper central ion instead of iron. Thus, the authors hypothesize that hemocyanin may have potential to inhibit RT-qPCR.

The purpose of this study is to observe and quantify the degree of inhibition that occurs from inhibitory compounds found in produce (pectin) and mollusks (hemocyanin, glycogen) by comparing Cq shifts. Each are thought to be inhibitory components found in produce and mollusks, respectively [5,24]. Bacteriophage MS2, an ssRNA phage and common norovirus surrogate, was chosen as a model (+)ssRNA genome to test the potential inhibitory effects to one step RT-qPCR, as it is widely available and easily cultivable [25].

2. Materials and methods

2.1. MS2 propagation and RNA extraction

The bacteriophage MS2 (15597-B1) was used as a model (+) ssRNA virus, as it has the advantage of being readily propagated to high levels in bacteria. *Escherichia coli* strain C-3000 (15597) was obtained from ATCC (Manassas, VA) and a plaque assay was conducted as described by manufacturer's instructions (ATCC 2018). MS2 stocks were filter sterilized and stored in −80°C until use. RNA was Trizol extracted according to manufacturer's instructions (Trizol Reagent, Invitrogen, Carlsbad, CA). All extracted RNA was aliquoted into minimal use volumes and stored at − 80 °C until use.

2.2. Generation of a MS2 RNA standard by RT-qPCR

The amplifiable genomic copes of the MS2 extract was determined by using an RNA standard curve as previously described [27]. Serial dilutions of the extracted MS2 RNA was amplified using the 632 F, 708R and 650 P primer-probe set [26] (Table 1) with NEB Luna® Universal One-Step RT-qPCR Kits (New England Biolabs, Ipswich, MA) and 3 μL of template. A 10-minute reverse transcription cycle was conducted at 55 °C, followed by enzyme inactivation at 95 °C for 10 min. Amplification was then performed for 45 cycles at 95 °C for 15 s and 60 °C for 60 s. At least two reactions were performed per sample with at least three separate reactions run.

Table 1
Real time RT-qPCR primer sequences.

Primer Name	Sequence (5'→ 3')	Location[a]
632F	GTC GCG GTA ATT GGC GC	632
708R	GGC CAC GTG TTT TGA TCG A	708
[b]650P	AGG CGC TCC GCT ACC TTG CCC T	650

Primer and probe selection were based on previously published data targeting the assembly protein gene of MS2 [26].
[a] Nucleotide corresponding to 5′ of primer of the *Escherichia* virus MS2 sequence (GenBank NC_001417).
[b] Probe was modified to include FAM at the 5' base and BHQ at the 3' base.

Table 2
Levels of PCR inhibitory compounds tested.

Pectin	Hemocyanin	Glycogen
0.25% (*m/V*)	0.25% (*m/V*)	10% (*m/V*)
0.125% (*m/V*)	0.125% (*m/V*)	5.0% (*m/V*)
0.0625% (*m/V*)	0.0625% (*m/V*)	2.5% (*m/V*)

2.3. Addition of inhibitory substance into RT-qPCR reactions

Pectin (93854), glycogen (G8751) and hemocyanin (H7017) were purchased from Sigma-Aldrich (St. Louis, MO) and stored according to manufacturer's instructions. Inhibitory compounds of interest were diluted into water solutions and added to RT-qPCR reactions in desired concentrations (Table 2). Serial dilutions of extracted RNA were then added to each desired well. The samples were then amplified with 632 F, 708R and 650 P (Table 1) using NEB Luna® Universal One-Step RT-qPCR Kit (New England Biolabs, Ipswich, MA). All reactions were performed in triplicate with no inhibitor and no template controls. Reactions used in gel electrophoresis analysis were cleaned using QIAquick PCR Purification Kit (QIAGEN, Hilden, Germany) and loaded on 2% agarose gels stained with 1× GelRed (Biotium) at 80 V for 1.5 h run time.

2.4. Data analysis

Statistical analysis was conducted in JMP using a connecting letters report. A p-value of less than 0.05 was considered significant. Negative reactions were considered results with a Cq value of 0.00. Negative reaction values (0.00) from replicates were not calculated into the average positive Cq values; Instead, replicates which displayed one negative reaction were indicated with a subscript "x", two negative reactions with a subscript "y" and three negative reactions with a subscript "z". The limit of detection (LOD) was calculated as the level for which no negative reactions were observed.

3. Results

The limit of detection for the MS2 RT-qPCR assay was found to be 10 PCR Units/reaction. Pectin was observed to cause significant inhibitory effects. Pectin at 0.25% was shown to cause the most severe effect, with complete inhibition (Fig. 1). Samples containing 0.125% pectin showed an average 7.44 ± 1.67 Cq difference when compared to the control at the highest dilution, 10^6 copies/reaction,

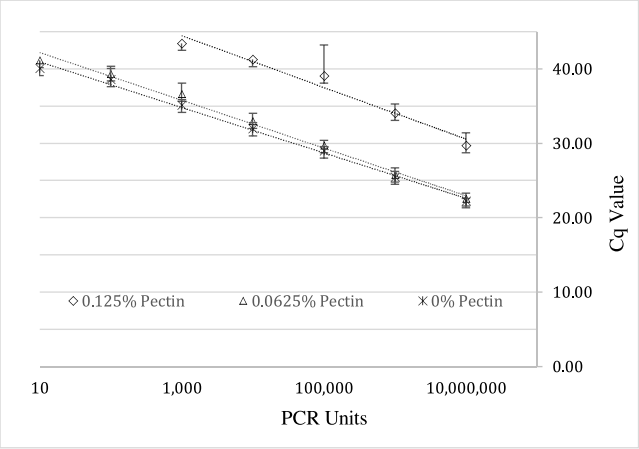

Fig. 1. Average Cq values of positive RT-qPCR reactions containing varying amounts of pectin. The percentage 0.25% is not shown, as the average Cq for all reactions was 0.
Data shown on the figure corresponds to the average of the positive values, reactions with no signal were excluded from the average calculations.

Table 3
Average difference between control Cq with samples contaminated with pectin.

PCR Units	0.25% (*m/V*)	0.125% (*m/V*)	0.0625% (*m/V*)
1,000,000	22.31 ± 0.30[a]	7.44 ± 1.67[b]	0.27 ± 0.79
100,000	25.52 ± 0.68[a]	8.57 ± 1.36[b]	0.26 ± 1.13
10,000	29.00 ± 0.55[a]	[x]10.09 ± 4.14[b]	0.72 ± 0.85
1000	32.04 ± 0.76[a]	[z]9.26 ± 3.47 [b]	0.97 ± 1.29
100	35.14 ± 0.77[a]	[z]8.33 ± 0.77[b]	1.49 ± 1.64
10	38.61 ± 1.46[a]	38.61 ± 1.46[a]	0.75 ± 1.76
1	40.08 ± 0.29[a]	40.08 ± 0.29[a]	[z]1.09 ± 0.33

[ab]Levels connected by letters are significantly different ($P < 0.05$) than 0% control. Those levels sharing the same letter are not significantly different for each column.
[y]Replicates with two negative reaction.
[x] Replicates with one negative reaction.
[z] Replicates with three negative reaction.

with negative reactions starting to occur at 10^4 (Table 3). No reactions at 0.125% reported a positive Cq after the 5th dilution (100 copies/reaction). There is a total average difference of 0.74 ± 1.11 Cq between the 0% control and 0.0625% pectin samples and were not found significantly different from one another. The 0.125% and 0.25% pectin samples have a total average difference of 13.72 ± 2.15 Cq and 30.44 ± 0.75 Cq when compared down to the detection limit (10 PCR units), as multiple reactions were completely inhibited (Table 3).

Glycogen was not found to cause any inhibitory effects up to 10% (*m/V*) (Supplemental Materials). Because of this, hemocyanin was investigated as a possible inhibitor present in mollusks. Interestingly, hemocyanin was found to cause significant inhibition at low levels. The apparent LOD for 0.25% (*m/V*) was found to be 10^6 copies/reaction (Table 5), after which there was complete inhibition at 10^3 copies/reaction. The detection limit of 0.125% hemocyanin was found to be 1000 PCR Units/reaction (Table 5). This concentration also began to encounter negative reactions at 100 copies/reaction with an average 9.32 ± 0.99 Cq increase when compared with the control at this dilution (Table 4). The 0.25% and 0.125% hemocyanin samples had significant differences from the 0% control, with average differences of 24.85 ± 0.39 Cq and 11.29 ± 0.47 Cq when compared down to the detection limit (10 PCR units), respectively. There is a total average difference of 1.65 ± 0.28 Cq between the 0% control and 0.0625% hemocyanin samples down to 10 PCR units, which were not found significantly different from one another (Fig. 2).

Table 4
Average difference between control Cq with samples with hemocyanin.

PCR Units	0.25% (*m/V*)	0.125% (*m/V*)	0.0625% (*m/V*)
1,000,000	13.44 ± 0.40[a]	3.25 ± 0.62 [a]	0.65 ± 0.09
100,000	[x]16.53 ± 0.13[a]	4.55 ± 0.22 [a]	1.16 ± 0.12
10,000	[z]14.77 ± 0.14[a]	5.09 ± 0.32 [a]	1.30 ± 0.10
1,000	31.80 ± 0.15[b]	7.52 ± 0.15[a]	1.88 ± 0.26
100	34.51 ± 0.99 [b]	[x]9.32 ± 0.99[a]	2.71 ± 0.90
10	38.04 ± 0.53 [b]	38.04 ± 0.53 [b]	2.17 ± 0.20
1	40.35 ± 0.00[b]	40.35 ± 0.00[b]	40.35 ± 0.00[b]

[ab]Levels connected by letters are significantly different ($P < 0.05$) than 0% control. Those levels sharing the same letter are not significantly different for each column.
[y]Replicates with two negative reaction.
[x] Replicates with one negative reaction.
[z] Replicates with three negative reaction.

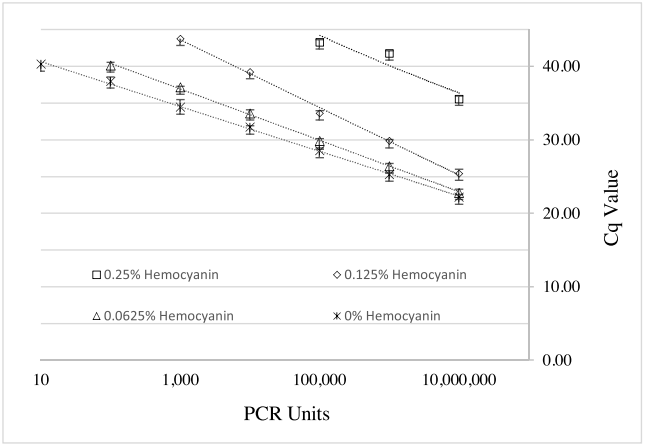

Fig. 2. Average Cq values of positive RT-qPCR reactions containing varying amounts of hemocyanin. Data shown on the figure corresponds to the average of the positive values, reactions with no signal were excluded from the average calculations.

Because previous research has shown hemoglobin to cause some form of fluorescence quenching, reactions testing all percentages of hemocyanin solutions with 100,000 and 10 PCR units were analyzed using agarose gel electrophoresis. Results from the gels did not differ from those from the RT-qPCR results at 0.125% (Supplemental Material). Unsurprisingly however, hemocyanin at 0.25% did not show up on the gel at 100,000 PCR units, as RT-PCR visualized via gel has lower sensitivity than probe-based RT-qPCR.

4. Discussion

In this study, pectin and hemocyanin were found to cause significant inhibition of one step RT-qPCR for bacteriophage MS2, a model (+)ssRNA virus. Pectin and hemocyanin had complete inhibition at 0.25% (*m/V*). Glycogen was not found to cause inhibition, even with levels as high as 10% (*m/V*) of reactions, despite having been previously implicated as being an inhibitor. All these molecules are present in foods commonly implicated in foodborne norovirus transmission; specifically produce and mollusks.

All hemocyanin reactions containing 1 PCR Units/reaction of RNA were found to be negative, minus a single reaction (the reason for a 0.00 standard deviation in Table 4) in the 0% control, which gave a Cq of 40.35 (data not shown). This MS2 assay was found to have a LOD of 10 PCR units/reaction, however sporadic detection is not uncommon for RT-qPCR reactions near their limit of detection. This can be seen throughout the paper, as many lower concentrations have replicates with negative results. Differences near the no inhibitor control limit of detection are not likely due to inhibition interactions, which can clearly be seen when comparing the 0% control and 0.0625% hemocyanin sample (Table 5).

Table 5
Limit of detection in PCR Units/reaction for samples contaminated with inhibitory substances.

0.25% pectin (*m/V*)	0.125% pectin (*m/V*)	0.0625% pectin (*m/V*)	0.0% pectin (*m/V*)	0.25% hemocyanin (*m/V*)	0.125% hemocyanin (*m/V*)	0.0625% hemocyanin (*m/V*)	0.0% hemocyanin (*m/V*)
N/A	100,000	10	10	1,000,000	1,000	10	10

Limit of detection was calculated as the level for which no negative reactions were observed.

Previous work has only reported pectin causing inhibition at higher concentrations than observed in this work. This could be due to multiple factors related to the previous reports, which used different PCR formulations, were not one-step real time RT-qPCR, and were not generally as analytically sensitive. It is possible that contemporary one-step RT-qPCR kits may be more prone to inhibition as the reverse transcriptase and real time probe are additional potential targets for inhibition. Future work identifying the degree to which the added reverse transcriptase step in the one-step assay renders the assay vulnerable to inhibition would be valuable. These results highlight the importance of pectinase in RNA extraction from produce, as little as 0.125% (*m/V*) can cause false negatives. Pectinase treatment has been found to work well, with reports of it aiding elimination of false-negative results in norovirus detection in produce [10,11]. However, the mechanism of pectin inhibition is still unknown. Previous reports have suggested that the acidic nature of pectin may cause the inhibition, as other acidic polysaccharides have also been observed to cause inhibition, including dextran sulphate and ghatti gum [12].

PCR inhibitors cause false negatives and higher detection limits. Common practice to remove inhibitors from norovirus samples has traditionally been through added steps in RNA extraction, however, carryover contamination is still common. This is especially true for compounds used in extraction, including phenols and alcohols. GlycoBlue (Invitrogen, Carlsbad, CA) is a popular coprecipitant used in many RNA extraction methods. It consists of blue dye covalently linked to glycogen derived from mussels, comparable to the glycogen tested in this study. Invitrogen has stated in their manuals that the addition of GlycoBlue should not disrupt RT-PCR reactions. This study further supports this claim, as the GlycoBlue used in RNA extraction is added to PCR in smaller amounts compared to the concentrations tested in this study. Further, the amount of glycogen found in oysters generally ranges from 4.6% to 17.1%, depending on age and season. Realistically, glycogen would not be found in PCR reactions higher than those tested in this study [15]. In all, these results suggest that glycogen is not likely a major contributor to the PCR inhibition observed with mollusk samples.

The goal of in-field viral detection of norovirus may be unobtainable using PCR technology. One of the many factors preventing this is the need to concentrate virus and perform RNA extraction to remove PCR inhibitors. However, some foods may have less inhibitory compounds compared to others and not require extensive purification. The levels of inhibitory compound used in this work range from those excess to what realistically would occur with direct use of food in reaction down to levels likely to be residual carryover after purification. For example, glycogen the highest concentration tested (10%), would be the equivalent of using direct oyster tissue as template in an oyster with 50% glycogen content—well beyond the amount of glycogen naturally found in oysters. Based on our observations, raspberries containing as little as 0.313% total pectin content or more could cause inhibition if directly used in reaction formulations as described. Raspberries have around 1% pectin, meaning that even crude sample extraction methods need an element to remove pectin to avoid inhibition. Further studies may be conducted on testing raw food samples vs only inhibitory compounds to observe the degree to which the compounds alone are responsible for inhibition.

Hemocyanin, a molecule found in the hemolymph of mollusks, was found to be inhibitory to RT-qPCR at very low concentrations. To the authors' knowledge, little has been known about which specific compounds in mollusk matrices cause PCR inhibition. This is important, as hemocyanin is the most abundant protein in mollusk hemolymph, and can be at levels as high as 85.6% of total hemolymph protein in some crustaceans [28]. In mollusks, hemocyanin functions similarly to hemoglobin—a well-documented PCR inhibitor in mammals. Unlike hemoglobin, a central copper group is found instead of an iron group. Free metal ions within a PCR solution are thought to cause reduction in the specificity of primers by replacement of magnesium. Hemoglobin was thought to release free iron ions and cause an inhibitory effect through this mechanism, but it was found that iron alone does not affect PCR mechanisms in the same way as hemoglobin. In fact, ionic iron and copper do not substantially inhibit PCR alone [29]. However, more recently, it was found that when using $FeCl_3$ and hemoglobin in PCR reactions, almost ten times the amount $FeCl_3$ was needed to cause a similar inhibitory effect as hemoglobin, though the specific mechanism by which this occurs is not known [30]. One hypothesis concludes that hemoglobin causes fluorescence quenching by binding dyes within the central cavity [30]. Although fluorophores were used in this paper, it should be noted that fluorescence quenching was not observed as the mechanism for the inhibition for hemocyanin, suggesting that the hemocyanin protein itself may be directly disrupting the enzymatic activity in RT-qPCR reactions. Hemocyanin differs in structure, having no central cavity or porphyrin rings as hemoglobin does. This is likely why no fluorescence quenching was observed, as the same degree of inhibition was observed with RT-qPCR signal and was confirmed using gel electrophoresis. Further studies should be conducted on more efficient ways to remove this compound, as well as the degree to which it may carryover through RNA extraction. Solvent based extractions can frequently result in carryover of protein if phenol is not homogenized or removed properly [31]. Column-based extraction methods traditionally result in less protein contamination, however, columns overloaded with sample can result in carryover. Both norovirus and hemocyanin are similar in diameter, being ~40 nm and 35 nm respectively [23,32], and would thus both be selected for with filtration techniques. Similarly, norovirus and hemocyanin have similar isoelectric points (pI), both being around 5–6 [32,33], meaning both may be precipitated into solution at similar pH.

5. Conclusion

In sum, this work quantifies the degree of inhibition that occurs from three compounds present in foods associated with norovirus transmission, demonstrating that pectin and hemocyanin should be considered when testing produce and mollusks. This information helps inform sample preparation for PCR-based detection of foodborne pathogens from produce and mollusks, as well as identifies a hitherto unreported specific inhibitory compound found in mollusks.

Declaration of Competing Interest

The authors declare no conflict of interest for this study.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.fshw.2019.09.002.

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