COMPARISON OF RNA EXTRACTION KITS FOR THE DETECTION OF MS2 COLIPHAGE ON GREEN ONIONS VIA RT-PCR

BY

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TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACKNOWLEDGEMENT</td>
<td>iii</td>
</tr>
<tr>
<td>LIST OF TABLES</td>
<td>vi</td>
</tr>
<tr>
<td>LIST OF FIGURES</td>
<td>vii</td>
</tr>
<tr>
<td>LIST OF SYMBOLS</td>
<td>viii</td>
</tr>
<tr>
<td>ABSTRACT</td>
<td>x</td>
</tr>
<tr>
<td>CHAPTER</td>
<td></td>
</tr>
<tr>
<td>1. INTRODUCTION</td>
<td>1</td>
</tr>
<tr>
<td>1.1 Introduction</td>
<td>1</td>
</tr>
<tr>
<td>1.2 Objectives of the study</td>
<td>4</td>
</tr>
<tr>
<td>2. LITERATURE REVIEW</td>
<td>5</td>
</tr>
<tr>
<td>2.1 Foodborne viruses</td>
<td>5</td>
</tr>
<tr>
<td>2.2 Transmission of the human enteric viruses</td>
<td>5</td>
</tr>
<tr>
<td>2.3 Routes of virus contamination</td>
<td>7</td>
</tr>
<tr>
<td>2.4 Foodborne outbreaks associated with green onions</td>
<td>8</td>
</tr>
<tr>
<td>2.5 Prevention and control of contamination</td>
<td>9</td>
</tr>
<tr>
<td>2.6 Detection of human enteric viruses in foods</td>
<td>10</td>
</tr>
<tr>
<td>2.7 Enteric bacteriophages as surrogates for mammalian viruses</td>
<td>21</td>
</tr>
<tr>
<td>2.8 The objectives of this study</td>
<td>23</td>
</tr>
<tr>
<td>3. MATERIALS AND METHODS</td>
<td>24</td>
</tr>
<tr>
<td>3.1 Materials</td>
<td>24</td>
</tr>
<tr>
<td>3.2 Comparison of RNA extraction methods for the detection of MS2 coliphage from green onions eluates</td>
<td>25</td>
</tr>
<tr>
<td>3.3 Comparison of RNA extraction methods for the detection of MS2 coliphage from concentrated green onions eluates</td>
<td>30</td>
</tr>
<tr>
<td>3.4 Comparison of Qiagen QIAamp Mini and MagMAX viral RNA extraction methods for the detection of MS2 coliphage from green onions</td>
<td>34</td>
</tr>
<tr>
<td>3.5 MS2 coliphage and Internal Control RNA quantification by real-time RT-PCR assay</td>
<td>36</td>
</tr>
<tr>
<td>3.6 Statistical analysis</td>
<td>37</td>
</tr>
</tbody>
</table>
4. RESULTS AND DISCUSSION .............................................. 40
   4.1 Comparison of RNA extraction methods for the detection of
       MS2 coliphage from green onions eluates .......................... 40
   4.2 Comparison of RNA extraction methods for the detection of
       MS2 coliphage from concentrated green onions eluates .......... 49
   4.3 Comparison of Qiagen QIAamp Mini and MagMAX viral RNA
       extraction methods for the detection of MS2 coliphage from
       green onions .................................................................................. 57

5. CONCLUSION AND RECOMMENDATIONS .............................. 62
   5.1 Conclusion .............................................................................. 62
   5.2 Recommendations ................................................................. 63

BIBLIOGRAPHY .............................................................................. 65
## LIST OF TABLES

<table>
<thead>
<tr>
<th>Table</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.1 Composition of PCR reaction</td>
<td>39</td>
</tr>
<tr>
<td>3.2 Sequences of primers and probes specific to genome of MS2 and Internal Control RNA in real-time RT-PCR assay</td>
<td>39</td>
</tr>
<tr>
<td>4.1 Comparison of four RNA extraction kits for detection of MS2 coliphage at inoculation level of $10^3$ pfu/ml by real-time RT-PCR</td>
<td>44</td>
</tr>
<tr>
<td>4.2 Comparison of four RNA extraction kits for detection of MS2 coliphage at inoculation level of $10^2$ pfu/ml by real-time RT-PCR</td>
<td>45</td>
</tr>
<tr>
<td>4.3 Comparison of four RNA extraction kits for detection of MS2 coliphage at inoculation level of $10^1$ pfu/ml by real-time RT-PCR</td>
<td>46</td>
</tr>
<tr>
<td>4.4 Comparison of four RNA extraction kits for detection of MS2 coliphage at inoculation level of $10^0$ pfu/ml by real-time RT-PCR</td>
<td>47</td>
</tr>
<tr>
<td>4.5 Limit of four RNA extraction kits for detection of MS2 coliphage at inoculation levels from $10^3$ to $10^0$ pfu/ml with respect to number of MS2 positive samples/total samples</td>
<td>48</td>
</tr>
<tr>
<td>4.6 Internal Amplification Control (IAC) assay results for four RNA extraction kits</td>
<td>48</td>
</tr>
<tr>
<td>4.7 Real-time RT-PCR detection of MS2 at 40 pfu/ml after eluate extraction by four RNA extraction kits with and without QIAshredder pre-treatment</td>
<td>56</td>
</tr>
<tr>
<td>4.8 Ten-fold dilution effect on reduction of inhibition of real-time RT-PCR for MagMAX kit with and without QIAshredder pre-treatment</td>
<td>56</td>
</tr>
<tr>
<td>4.9 Limit of Qiagen QIAamp Mini and MagMAX viral RNA extraction kits for detection of MS2 at low inoculation levels (20 and 5 pfu/g)</td>
<td>61</td>
</tr>
</tbody>
</table>
## LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>4.1 Comparison of real-time RT-PCR cycle thresholds ($C_t$) for detection of MS2 and IAC targets after Lot A concentrated eluates were extracted with and without QIAshredder pre-treatment</td>
<td>53</td>
</tr>
<tr>
<td>4.2 Comparison of real-time RT-PCR cycle thresholds ($C_t$) for detection of MS2 and IAC targets after Lot B concentrated eluates were extracted with and without QIAshredder pre-treatment</td>
<td>53</td>
</tr>
<tr>
<td>4.3 Comparison of real-time RT-PCR cycle thresholds ($C_t$) for detection of MS2 and IAC targets after Lot C concentrated eluates were extracted with and without QIAshredder pre-treatment</td>
<td>54</td>
</tr>
<tr>
<td>4.4 Comparison of real-time RT-PCR cycle thresholds ($C_t$) for detection of MS2 and IAC targets after Lot D concentrated eluates were extracted with and without QIAshredder pre-treatment</td>
<td>54</td>
</tr>
<tr>
<td>4.5 Comparison of real-time RT-PCR cycle thresholds ($C_t$) for detection of MS2 and IAC targets after Lot E concentrated eluates were extracted with and without QIAshredder pre-treatment</td>
<td>55</td>
</tr>
<tr>
<td>4.6 Comparison of real-time RT-PCR cycle thresholds ($C_t$) for detection of MS2 and IAC targets after onion Lot A was extracted with and without QIAshredder pre-treatment</td>
<td>60</td>
</tr>
<tr>
<td>4.7 Comparison of real-time RT-PCR cycle thresholds ($C_t$) for detection of MS2 and IAC targets after onion Lot B was extracted with and without QIAshredder pre-treatment</td>
<td>60</td>
</tr>
</tbody>
</table>
LIST OF SYMBOLS

<table>
<thead>
<tr>
<th>Symbol</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>CDC</td>
<td>Centers for Disease Control and Prevention</td>
</tr>
<tr>
<td>cDNA</td>
<td>Complementary Deoxyribonucleic Acid</td>
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<tr>
<td>CPEs</td>
<td>Cytopathic Effects</td>
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<tr>
<td>C&lt;sub&gt;t&lt;/sub&gt;</td>
<td>Cycle Threshold</td>
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<td>CTAB</td>
<td>Cetyltrimethylammonium Bromide</td>
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<tr>
<td>DNA</td>
<td>Deoxyribonucleic Acid</td>
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<tr>
<td>dsDNA</td>
<td>Double-stranded DNA</td>
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<tr>
<td>FCV</td>
<td>Feline Calicivirus</td>
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<td>FDA</td>
<td>U.S. Food and Drug Administration</td>
</tr>
<tr>
<td>GAP</td>
<td>Good Agriculture Practice</td>
</tr>
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<td>GMP</td>
<td>Good Manufacturing Practice</td>
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<td>HACCP</td>
<td>Hazard Analysis and Critical Control Point</td>
</tr>
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<td>HAV</td>
<td>Hepatitis A Virus</td>
</tr>
<tr>
<td>IAC</td>
<td>Internal Amplification Control</td>
</tr>
<tr>
<td>MNV</td>
<td>Murine Norovirus</td>
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<tr>
<td>MPN</td>
<td>Most Probably Number</td>
</tr>
<tr>
<td>Nov</td>
<td>Norovirus</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate Buffered Saline</td>
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<tr>
<td>PEG</td>
<td>Polyethylene Glycol</td>
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<tr>
<td>pfu</td>
<td>Plaque Forming Units</td>
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<tr>
<td>PV</td>
<td>Poliovirus</td>
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<tr>
<td>qPCR</td>
<td>Quantitative Polymerase Chain Reaction</td>
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<tr>
<td>RNA</td>
<td>Ribonucleic Acid</td>
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<tr>
<td>rpm</td>
<td>Revolutions Per Minute</td>
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<tr>
<td>RTE</td>
<td>Ready-to-eat</td>
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<tr>
<td>RT-PCR</td>
<td>Reverse Transcriptase-polymerase Chain Reaction</td>
</tr>
<tr>
<td>RV</td>
<td>Rotavirus</td>
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<tr>
<td>TCID$_{50}$</td>
<td>50% Tissue Culture Infective Dose</td>
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</tr>
<tr>
<td>TSBYE</td>
<td>Tryptic Soy Broth Yeast Extract</td>
</tr>
</tbody>
</table>
ABSTRACT

Real-time reverse transcriptase-polymerase chain reaction (RT-PCR) offers a rapid and sensitive molecular method for detection of enteric viruses. Unfortunately, these assays are often hampered by the low numbers of viruses found in foods and PCR inhibition due to matrix carryover after RNA extraction. The purpose of this research was to compare the ability of four RNA extraction kits to extract and purify MS2 bacteriophage RNA from (1) green onion eluates and (2) concentrated eluates prepared with and without Qiagen QIAshredder pre-treatment, using detection by real-time RT-PCR.

Green onion eluates inoculated at $10^3$ to $10^0$ pfu/ml or concentrated eluates inoculated at 40 pfu/ml, with and without Qiagen QIAshredder processing, were evaluated. Overall, the detection from each sample varied with the kits and degree of inhibition allowed, with inhibition generally following MagMAX > UltraSens > Mo Bio > QIAmp Mini. The addition of the QIAshredder reduced the inhibition from the MagMAX kit, increasing the samples detected from 1/5 to 5/5. The Qiagen QIAamp Mini and MagMAX kits were further tested by comparing extractions from green onions inoculated at 20 and 5 pfu/g, with and without QIAshredder pre-treatment. QIAamp Mini allowed detection of 2/2 inoculated at 20 and 5 pfu/g regardless of QIAshredder, whereas the MagMAX required the QIAshredder to detect 1 of 2 at 20 pfu/g. MagMAX did not allow detection of the MS2 at 5 pfu/g even with the use of the QIAshredder. Overall, the Qiagen QIAamp Viral Mini kit is recommended for extraction of MS2 coliphage from green onions, however all kits would be useful with the addition of the QIAshredder to reduce inhibition.
CHAPTER 1
INTRODUCTION

1.1 Introduction

Recent epidemiological evidence indicates that human enteric viruses, in particular noroviruses (NoV), which cause acute gastroenteritis and other viruses such as hepatitis A virus (HAV) and rotavirus (RV), are recognized as the most common cause of foodborne diseases in the U.S. and worldwide (Cliver, 1997; Koopmans & Duizer, 2004; Sair, D’Souza, & Jaykus, 2002a). The major risk factor for enteric viral illnesses is associated with the consumption of raw or insufficiently cooked shellfish (Grohmann, Murphy, Christopher, Auty, & Greenberg, 1981; Le Guyader et al., 2008; Power & Collins, 1989). Additionally, fruit, such as minimally processed berries (Calder et al., 2003; Gaulin, Ramsay, Cardinal, & D’Halevyn, 1999; Le Guyader et al., 2004; Niu et al., 1992), and vegetables including ready-to-eat salads, lettuce, and green onions, have also been reported as the cause of foodborne outbreaks (Dentinger et al., 2001; Long, Adak, O’Brien, & Gillespie, 2002; Rosenblum, Mirkin, Allen, Safford, & Hadler, 1990). An outbreak of hepatitis A virus in western Pennsylvania due to the ingestion of contaminated green onions resulted in three deaths among a total of 601 cases (Wheeler et al., 2005).

Foodborne viruses can be transmitted enterically by a fecal-oral cycle in a wide variety of manners (Bosch, Pintó, & Abad, 2006). Enteric viral contamination of food products may occur at any point during planting, harvesting, processing, storage, distribution, or final preparation by two main routes: (i) contact with human sewage polluted water or soil and irrigating with virus-contaminated wastewater, and (ii)
handling by virus-infected individuals with poor hygiene during harvest, packaging and
preparation (Butot, Putallaz, Amoroso, & Sánchez, 2009; Cheong et al., 2009; Fiore,
2004). Several disinfection technologies including washing with chlorinated water
(Keswick et al., 1985; Li, Xin, Wang, Zheng, & Chao, 2002), high hydrostatic pressure
(Kingsley, Hoover, Papafragkou, & Richards, 2002), gamma irradiation (Bidawid, Farber,
& Sattar, 2000) and thermal processing (Parry & Mortimer, 1984) have been highly
investigated for reducing or eliminating foodborne viruses in water, fruits, vegetables,
and shellfish. In addition, to reduce foodborne transmission of viruses it is possible to
apply food safety management systems, such as Hazard Analysis and Critical Control
Point (HACCP) and Good Manufacturing Practice (GMP) (Fiore, 2004; Koopmans &
Duizer, 2004).

Detection of viral contamination of food or water has been problematic. The
viruses of greatest concern, HAV and NoV, do not replicate in the environment, water or
food. Moreover, viral loads presented in food samples are typically much lower than
those found in clinical samples, therefore, the detection methods need to be sensitive
(Butot, Putallaz, & Sánchez, 2007; Sair, D'Souza, Moe, & Jaykus, 2002b). Classical
detection of human enteric virus from food concentrates is based on mammalian cell
culture assays; nevertheless, the fundamental drawback of this method is that besides
being costly, laborious and time-consuming, some enteric viruses grow poorly (HAV) or
not at all (NoV) in cultured cells (Downes & Ito, 2001; Fong & Lipp, 2005; Haramoto,
Katayama, & Ohgaki, 2004; Jaykus, 2000b). For these reasons, real-time reverse
transcriptase-polymerase chain reaction (RT-PCR) has been utilized as a rapid, sensitive
and reliable tool for the detection and quantification of HAV, NoV and other enteric
viruses in food samples (Casas, Amarita, & de Marañón, 2007; Costafreda, Bosch, & Pinto, 2006; Dubois et al., 2006; Jothikumar et al., 2005; Shan, Wolffs, & Griffiths, 2005).

The application of real-time RT-PCR for enteric RNA virus detection in food is hindered by two challenges. The first is the need to concentrate low levels of viruses from complex food matrices into a volume that is small enough for real-time RT-PCR analysis (Butot et al., 2007). This may be overcome by the use of concentration techniques such as ultracentrifugation. The second issue is the need to remove or overcome inhibition of the real-time RT-PCR, which may originate from constituents of bacterial cells and non-target DNA, unintentional contamination with reagents, containers, or disposables during reaction preparation, or from food constituents including organic and phenolic compounds, glycogen, fats, and calcium compounds (Wilson, 1997) co-extracted with viral RNA and in turn often leads to false-negative RT-PCR results (Butot et al., 2007; Croci et al., 2008).

Sample processing for fresh produce is one of the critical steps requiring evaluation to determine the techniques which allow the best recovery of viral RNA without carryover of inhibitory compounds into real-time RT-PCR. A range of extraction protocols have been employed for extracting enteric viral RNA and simultaneously removing or inactivating potential inhibitors from complex food matrices prior to RT-PCR (Butot et al., 2007; Fong & Lipp, 2005; Lampel, Orlandi, & Kornegay, 2000; Sair et al., 2002b); however, many of them depend on the use of complex home-made reagents or on procedures that are not commercially available (Bianchi, Dal Vecchio, Vilarino, & Romalde, 2011). A wide variety of methods using commercial kits for the rapid isolation
and purification of nucleic acids from different sources offer a reliable and reproducible means of obtaining enteric viral RNA for detection by RT-PCR. Most of these kits are based on guanidinium lysis, followed by capture of nucleic acids on a column or bead of silica (Bosch et al., 2011). Several publications have evaluated and compared various kits’ abilities for extraction of virus RNA from different matrices such as water (Burgener, Candrian, & Gilgen, 2003), strawberries (Bianchi et al., 2011) or fecal specimens (Hale, Green, & Brown, 1996), but none has been validated for comparison of their purification efficiency in concentrated green onion rinses.

1.2 Objectives of the Study

This current study evaluated the performance of four commercial RNA extraction kits for RNA isolation and removal of inhibitors from green onion extracts prior to real-time RT-PCR detection using MS2 coliphage as an enteric virus surrogate. The kits evaluated were: Qiagen QIAamp Viral RNA Mini Kit and QIAamp UltraSens Virus Kit (Qiagen Inc., Valencia, CA), Mo Bio UltraClean Tissue & Cells RNA Isolation Kit (Mo Bio Laboratories, Inc., Carlsbad, CA), and Ambion MagMAX Viral RNA Isolation Kit (Ambion Inc., Austin, TX). The aim of this work was to compare the ability of four commercially available nucleic acid extraction kits to extract and purify MS2 coliphage RNA from (1) green onion eluates, and (2) ultracentrifuge-concentrated eluates prepared with and without Qiagen QIAshredder pre-treatment, using detection by real-time RT-PCR.
2.1 Foodborne Viruses

Viruses, having a diameter between 15 to 400 nm, are tiny microorganisms resulting in a wide range of diseases in plant, animals and humans (Koopmans & Duizer, 2004). The most relevant in foodborne virus infections are viral gastroenteritis caused by small round structured viruses, rotavirus, astrovirus and parvovirus, and enterically transmitted hepatitis viruses including hepatitis A and hepatitis E viruses (Appleton, 2000). According to recent estimates of foodborne illness by Centers for Disease Control and Prevention (Scallan et al., 2011), among foodborne pathogens, virus contaminated food accounted for 59% of illnesses, 27% of hospitalizations and 12% of deaths in the United States during the years from 2000 to 2008. Recent epidemiological evidence has shown that human enteric viruses are recognized as the most common cause of foodborne diseases in the U.S. and worldwide (Cliver, 1997; Sair, D’Souza, & Jaykus, 2002a; Scallan et al., 2011).

2.2 Transmission of the Human Enteric Viruses

Viruses can be transmitted by a fecal-oral cycle in a variety of ways, including person-to-person contact, vector transmission by flies or fomites, and vehicle transmission via food and water (Bosch, Pintó, & Abad, 2006; Cliver, 1997). Patients suffering from diarrhea or hepatitis may excrete as many as $10^5$ to $10^{11}$ virus particles per gram of stool (Farthing, 1989). In addition, a single episode of vomit of a patient with norovirus-induced gastroenteritis may shed around $10^5$-$10^9$ particles per gram of stool, but may be as high as $10^{11}$/g (Lopman et al., 2012). Human enteric viruses transmission
via foods has seems been a frequent occurrence. Many foods but molluscan shellfish in particular, may be contaminated by fecally polluted water in their growing and harvesting areas. Additionally, foods such as ready-to-eat (RTE) and prepared foods may also be contaminated as a result of poor personal hygiene (Appleton, 2000; Jaykus, 2000b).

Foodborne virus infections are often associated with the consumption of bivalve molluscan shellfish (clams, cockles, mussels, and oysters) which are filter feeders capable of collecting viruses and other pathogens from human fecal contaminated waters. These viruses may be harbored for days or weeks in the digestive tract of the shellfish (Grohmann, Murphy, Christopher, Auty, & Greenberg, 1981; Power & Collins, 1989; Sair et al., 2002a). Unlike many other seafoods, shellfish are usually eaten raw or lightly cooked with their digestive tracts in place. Moreover, research by DiGirolamo, Liston, and Matches (1970) has indicated that viruses in oysters may withstand thermal inactivation during cooking. Viral gastroenteritis associated with the consumption of shellfish in Hawaii in 1991 has been demonstrated (Centers for Disease Control and Prevention [CDC], 1991); and a large foodborne disease outbreak of hepatitis A in Shanghai, China in 1988 has also been associated with the ingestion of raw clams (Halliday et al., 1991).

Populations may also be infected by the consumption of crop materials (Metcalf, Melnick, & Estes, 1995), raw vegetables (spinach), and fruits (strawberries) that have been grown in fields irrigated with wastewater, or fertilized with human feces and conditioned with inadequately decontaminated sewage sludge (Hutin et al., 1999; Niu et al., 1992). Meanwhile, outbreaks of viral infection due to poor personal hygiene of food handlers are increasingly reported for ready-to-eat foods, such as lettuce, salad vegetables,
sandwiches and bakery products (Cliver, 1997; Rosenblum, Mirkin, Allen, Safford, & Hadler, 1990). A previous example is an outbreak of hepatitis A virus associated with consumption of a variety of gourmet foods prepared by an infected food handler in Denver, Colorado in 1992 in which nearly 5,000 people were affected (Dalton, Haddix, Hoffman, & Mast, 1996).

### 2.3 Routes of Virus Contamination

Viral contamination of food products may occur at any point during cultivation, harvesting, or during processing, storage, distribution, or final preparation (Butot, Putallaz, Amoroso, & Sánchez, 2009; Fiore, 2004). Pre-harvest contamination may occur in foods by cultivation in human feces-polluted soil, irrigation with inadequately treated wastewater and/or fertilizers contaminated with sewage (Bosch et al., 2006). Furthermore, foods can potentially become contaminated from handling by virus-infected individuals during harvesting, washing through infected water, and hydrocooling with virus-contaminated ice during storage and shipping (Appleton, 2000; Gerba & Choi, 2006; Koopmans, Von Bonsdorff, Vinjé, De Medici, & Monroe, 2002). During the preparation and distribution, infected food handlers who practice poor personal hygiene have also been identified as an important role for contamination of ready-to-eat foods (Hedberg & Osterholm, 1993), especially when handing cooked products with bare hands (Bryan, 1996). Fecal material deposited on hands or under nails may come in contact with food products as a result of poor hand-washing practices after toilet use (Jaykus, 2000b). Enteric virus can be shed by infected food handlers for long periods of time, even after full recovery from a symptomatic state (Parashar et al., 1998), and survive for periods exceeding the shelf-lives of the products (Butot et al., 2009).
2.4 Foodborne Outbreaks Associated with Green Onions

An outbreak of hepatitis A virus in western Pennsylvania was attributed to the consumption of contaminated green onions from Mexico added to the restaurant’s homemade salsa. This outbreak affected more than 600 people and resulted in three fatalities (Wheeler et al., 2005). In two other foodborne outbreaks of hepatitis A, including 43 cases in Ohio, 1998 (Dentinger et al., 2001) and 31 cases in Kentucky and Florida, 2000 (Datta, 2001), fresh green onions were suspected of being contaminated before retail distribution. Green onions have also been implicated in outbreaks of *Shigella flexneri* (Cook et al., 1995) and *Cryptosporidium* species infections (CDC, 1998).

Green onions, which are multilayered and may retain soil particles that could harbor fecal contaminants, are probably contaminated during planting, irrigating, harvesting, processing, or shipping. During harvesting, green onions require extensive handling; therefore, contamination may be most likely to occur at this step. Alternatively, virus-infected water used for irrigation, processing, and hydrocooling or ice that is layered on top of the boxed green onions before shipping may have been the potential source of contamination (Bosch et al., 2006; Dentinger et al., 2001). In order to achieve sensitive detection of enteric virus on green onions, Guévremont, Brassard, Houde, Simard, and Trottier (2006) developed a viral elution-concentration method and compared RT-PCR primer systems for the detection of hepatitis A virus and norovirus in contaminated green onions. This method would be useful for diagnostic laboratories to perform viral analyses of fresh products in cases of foodborne contaminations. Shan, Wolffs, and Griffiths (2005) have also applied an immunomagnetic capture method and a real-time reverse transcription-PCR assay to quantify hepatitis A virus in green onion
HAV can be detected at as low as 0.5 PFU in produce rinses and concentrated 20-fold by this combined protocol.

2.5 Prevention and Control of Contamination

Epidemiological data of numerous outbreaks has clearly demonstrated that any food can act as an efficient vehicle for the transmission of human enteric viruses by the fecal to oral route (Sair et al., 2002a). Various control strategies have been developed and were found to efficiently prevent foodborne transmission of diseases in water, fresh fruits and vegetables. Viral infections via hand contamination by food handlers during food manufacturing operations have obviously been common problems, so there is a need for instituting good hygiene practices in the field, kitchen and serving areas. Sanitary facilities and hand washing stations should be provided for field workers and employees. Hygiene training and education, such as proper hand washing (using friction action and a nail brush) and glove wearing for food handlers is also necessary to prevent contamination of food products (Ansari, Sattar, Springthorpe, Wells, & Tostowaryk, 1989; Cliver & Kostenbader, 1984).

Viruses in foods may be inactivated before consumption if contamination has not been prevented. Studies have evaluated the efficiency of various disinfection modalities, including chlorinated water (Keswick et al., 1985; Li, Xin, Wang, Zheng, & Chao, 2002), high hydrostatic pressure (Kingsley, Hoover, Papafragkou, & Richards, 2002), gamma irradiation (Bidawid, Farber, & Sattar, 2000) and thermal processing (Parry & Mortimer, 1984) for reducing or eliminating foodborne viruses in water, vegetables and fruits, and shellfish. Other commonly applied processes such as washing with different sanitizers, freezing, and low pH treatment are less effective at removing or disinfecting foods
contaminated with enteric viruses (Appleton, 2000; Butot, Putallaz, & Sánchez, 2008). Additionally, reducing foodborne transmission of viruses can be achieved by using food safety management systems (HACCP, GAP and GMP), safety guidelines, and best-practice documents similar to those recommended for reducing contamination by other foodborne pathogens before or during food manufacturing processes (Fiore, 2004; Koopmans & Duizer, 2004).

2.6 Detection of Human Enteric Viruses in Foods

2.6.1 Virus Concentration Methods. Detection of enteric viruses in food or water has been problematic. The viruses of greatest health concern, hepatitis A and the Noroviruses, do not replicate in the environment, water or food and are usually present at very low levels. Therefore, the detection methods need to be very sensitive (Sair, D’Souza, Moe, & Jaykus, 2002a). It is often necessary to concentrate and purify the viruses into a volume and quality compatible with detection methods (Jaykus, De Leon, & Sobsey, 1996). Applicable concentration techniques include precipitation, adsorption-elution, ultrafiltration, and ultracentrifugation (Cliver, 1997).

There are several precipitation methods including flocculation and solvent extraction, but the most common is the polyethylene glycol (PEG) precipitation method. Polyethylene glycol (PEG), identified as a chemical inert, nontoxic, water-soluble synthetic polymer (Lewis & Metcalf, 1988), has been applied in aqueous polymer two-phase systems that are helpful for viruses concentration and isolation (Albertsson, 1960). Some articles have described the application of PEG precipitation for concentrating and purifying human enteric viruses from a variety of food samples including oysters (Jaykus et al., 1996), fresh-cut vegetables (Sánchez, Elizaquível, & Aznar, 2012), and hamburger
Advantages of the PEG concentration method, in addition to being rapid, inexpensive, and gentle on viruses, include the ability to precipitate viruses at neutral pH and in high ionic concentrations in the absence of other organic material (Lewis & Metcalf, 1988).

The adsorption-elution technique is currently regarded as one of the most useful methods for concentrating viruses in water (Eaton, Clesceri, & Greenberg, 1995). Adsorption filtration can employ nitrocellulose membranes - either electropositive or electronegative charged microporous filters (0.2-0.45 mm) - to adsorb viruses to filters that have a pore size that is larger than the viral particles (Eaton et al., 1995; Hou, Gerba, Goyal, & Zerda, 1980). Adsorption of viral particles to the filter is obtained by the electrostatic interactions between the viral particles and the filter (Eaton et al., 1995; Hou et al., 1980; Shields & Farrah, 1983). At ambient pH, most enteric viruses are captured by electropositive filter media since they are negatively charged (Water, 2010). Enteric viruses are also known to adsorb to negatively charged membranes efficiently in the presence of magnesium chloride (Sobsey, 1995; Wallis & Melnick, 1967) or other multivalent cation salts, or under acidic pH conditions (Sobsey, Wallis, Hendersen, & Melnick, 1973). Adsorbed viruses are then eluted with a small volume of alkaline solution such as beef extract, glycine, tryptose phosphate buffer or sodium hydroxide, which release viruses back into solution by altering the surface charge of the viral particles (Brassard, Seyer, Houde, Simard, & Trottier, 2005; Sobsey & Jones, 1979; Villar, de Paula, Diniz-Mendes, Lampe, & Gaspar, 2006; Water, 2010). This method has been applied to viruses in large volumes of tap water (Nupen & Bateman, 1985; Sobsey, Oglesbee, Wait, & Cuenca, 1985), groundwater (Abbaszadegan, Huber, Gerba, & Pepper,

Ultrafiltration is a method that has been used for concentration of microbes, including viruses in water, since the 1970’s (Belfort, Rotem, & Katzenelson, 1975). Ultrafiltration is based on size-exclusion where molecules smaller than the pore size of the filter pass through the membrane and out of the system and with larger particles concentrated in the retentate (Morales-Morales et al., 2003). Virus research has focused on concentrating viruses from tap (Hill et al., 2007) and distilled water (Garin, Fuchs, Bartoli, & Aymard, 1996) by ultrafiltration, however there is some work investigating the use of ultrafiltration for rapid concentration of enteric viruses from berries and vegetables (Butot, Putallaz, & Sánchez, 2007). Ultrafiltration offers the advantages of not requiring an elution step and is less affected by pH (Olszewski, Winona, & Oshima, 2005); however, it does have some disadvantages. The pore size has to be small enough to retain viruses and thus filters can become clogged which reduces the flow rate while dealing with large volumes of water (Oshima, 1998).

Ultracentrifugation is a process that uses centrifugal force to separate and purify mixtures of biological particles in a liquid medium based on size, shape, and density difference between the particles and the liquid medium in which these are suspended. In a solution, particles whose density is higher than that of the solvent may sink as sediment and particles that are lighter may float to the top when they undergo acceleration in a centrifugal field. Ultracentrifugation is carried out in two ways: analytical and preparative ultracentrifugation. Analytical ultracentrifugation aims to purify macromolecules or
isolate supramolecular assemblies; preparative ultracentrifugation is concerned with the actual separation of tissues, cells, subcellular structures, membrane vesicles and viruses (Koolman & Roehm, 2005; Wilson & Walker, 2010). A general feature in all centrifuges is the central motor that spins a rotor containing the samples to be separated. Particles of biochemical interest are usually dispersed in a liquid buffer system contained in specific tubes or separation chambers that are located in specialized rotors. Preparative ultracentrifuges can be operated at relative centrifugal force of up to 900,000 × gravity. The rotor chamber should be sealed, evacuated and refrigerated to minimize excessive rotor temperatures generated by frictional resistance between the spinning rotor and air (Wilson & Walker, 2010). Ultracentrifugation has most commonly been used for concentrating and purifying enteric viruses from a variety of foods including shellfish (Sunen, Casas, Moreno, & Zigorraga, 2004), and lettuce (Rutjes, Lodder-Verschoor, van der Poel, van Duijnhoven, & Husmani, 2006). This method has also been adapted for isolating enteric viruses from sewage (Mack, Mallmann, Bloom, & Krueger, 1958) and wastewater samples (Nordgren, Matussek, Mattsson, Svensson, & Lindgren, 2009). Ultracentrifugation requires expensive equipment and can be used only with eluates free of vegetable matter that are obtained from hard fruits or vegetable (Croci et al., 2008).

2.6.2 Virus Detection Methods.

2.6.2.1 Mammalian Cell Culture Assays. Historically, conventional methods for the detection of human enteric viruses from concentrates of food samples have largely depended on the infectivity of the viruses for susceptible live laboratory hosts (Jaykus, 2000a). Common cell lines specifically used for each virus type for quantification and isolation of culturable human enteric viruses include the BGMK (buffalo green monkey
kidney-derived) cells, MA104 (rhesus monkey kidney-derived) cells, RD (human rhabdomyosarcoma-derived) cells, MDBK (Madin-Darby bovine kidney) cells, PK-15 A (pig kidney) cells, and HeLa (human cervical carcinoma) cells (Doherty, Todd, McFerran, & Hoey, 1999; Jaykus, 2000a; Ley, Higgins, & Fayer, 2002; Schmidt, Ho, Riggs, & Lennette, 1978). Virus detection by cell culture assay (TCID\textsubscript{50}, tissue culture infective dose, or MPN) relies mainly on the formation of cytopathic effects (CPEs) observable under a light microscope, followed by quantification of the viruses by plaque assay (Bosch et al., 2011). For hepatitis A virus, fetal rhesus monkey kidney-derived (FRhK-4) cells are used routinely to propagate the HM-175 lab-adapted strain. This tissue culture line is generally ineffective for the detection of wild-type HAV which cannot induce cytopathic effects (CPEs) in these permissive cells (Cromeans, Sobsey, & Fields, 1987; De Leon & Jaykus, 1997).

The fundamental drawback to cell culture assays is that they are costly, laborious and time-consuming - often requiring a period of days to weeks to finally identify cytopathic effects (CPEs), or obvious cell death (Fong & Lipp, 2005; Jaykus, 2000a). Additionally, some samples may be cytotoxic but appear as cytopathic effects (CPEs) on cells; two or more viruses may potentially present in the same inoculum; and viruses that replicate more slowly may fail to be detected if differential rates of growth occur (Fong & Lipp, 2005; Jaykus, 2000a). A single universal cell culture system for detecting a majority of the numerous enteric viruses has not been established; therefore there are limitations for replication and detection of many viruses through cell culture (Chapron, Ballester, Fontaine, Frades, & Margolin, 2000). For example, hepatitis A virus may be propagated in cell cultures, but it grows very slowly and usually without visual cytopathic
effects, making its detection by the plaque assay technique impossible (Downes & Ito, 2001). Likewise, noroviruses, one of the major causative agents for gastroenteritis and foodborne outbreaks, do not replicate in conventional cell or tissue culture, thus they cannot be assayed by cell culture techniques (Haramoto, Katayama, & Ohgaki, 2004).

### 2.6.2.2 Immunological Methods

As a result of the technological limitation of mammalian cell culture infectivity assays, immunological methods based on the antigenic specificity of the viral coat protein have been used as an alternative detection strategy for the enteric viruses for which no host system exists (Jaykus, 2000; Sair et al., 2002a). Early work focused on applying immunological techniques for detection of viruses like HAV in shellfish (Sobsey, 1985). Additionally, a number of the clinical immunological methods illustrated in the articles have indeed been adapted for the detection of Noroviruses in clinical specimens associated with foodborne disease outbreaks (Hedberg & Osterholm, 1993; Heun, Vogt, Hudson, Parren, & Gary, 1987; Lewis, Ando, Humphrey, Monroe, & Glass, 1995). However, while these techniques are effective for clinical specimens, the general unavailability of reagents and inadequate assay detection limits (>10⁵ infectious virus particles) compromise their practical application for the detection of viral contamination of foods (Parker, Cubitt, Jiang, & Estes, 1993).

### 2.6.2.3 Nucleic Acid Hybridization Methods

Gene probes and nucleic acid hybridization techniques, both radioactive and nonradioactive, have been employed to detect human enteric viruses in clinical, environmental, and food samples (Bosch, Gajardo, Diez, & Pintó, 1996; Jaykus, 2000a; Jaykus, Hemard, & Sobsey, 1994). Detection limits for hybridization to genomic viral RNA have been reported at 500 to 1000 infectious units for HAV (Shieh et al., 1991), 2.5×10⁵ physical particles of rotavirus
16

SA-11 (Dimitrov, Graham, & Estes, 1985), and 500 to 1000 plaque forming units (PFU) of coxsackievirus B3 enterovirus (Jaykus et al., 1994), respectively. In general, single-stranded RNA probes used to detect HAV in stool and water samples are 5 to 8-fold more sensitive than cDNA probes (Jiang, Estes, & Metcalf, 1987; Shieh et al., 1991). Gene probes have been used to detect human enteroviruses (Margolin, Richardson, DeLeon, & Gerba, 1989), HAV (Jiang et al., 1987), and rotaviruses (Zhou, Estes, Jiang, & Metcalf, 1991) in environmental waters and shellfish (Jaykus, 2000a). These hybridization methods still have significant limitations in that the detection limit often exceeds $10^3$ to $10^4$ physical particles/sample (Sair et al., 2002b).

2.6.2.4 Nucleic Acid Amplification Methods. The molecular-based polymerase chain reaction (PCR) assays for detecting enteric viral pathogens have successfully been utilized for a number of years. Conventional PCR methodology uses a pair of oligonucleotides or primers, each hybridizing to one strand of a double-stranded DNA (dsDNA) target. Three types of primers (random primers, polythymine primers, and specific primers) are commonly used as a substrate for DNA polymerase which creates a complementary strand by the way of sequential addition of deoxynucleotides (Woods, 2013). A typical cycle of PCR can be summarized as follows: dsDNA is denatured and separated by an increase in temperature. As temperatures decrease, the primers begin to anneal to the separated DNA, the extension of the DNA strand with the primers occurs through addition of deoxynucleotides (Gerba, Reynolds, Dowd, & Pepper, 2001). The final PCR product may be analyzed by agarose gel electrophoresis in the presence of ethidium bromide in which the correct size of the product can be examined visually by ultraviolet light (Woods, 2013). For RNA viruses like enteroviruses, NoV, and HAV, it is
necessary to convert viral RNA to cDNA prior to PCR (Rotbart, 1990). During reverse transcription, a primer is required for the reverse transcriptase (RNA-dependent DNA polymerase) to initiate the synthesis of a cDNA from the viral RNA (Rodriguez, Pepper, & Gerba, 2009).

Most foodborne viruses contain RNA rather than DNA. Real-time reverse transcriptase-PCR (RT-PCR) assays are widely used in the field of food virology and are continuously evolving due to its high speed, sensitivity, reproducibility and minimization of contamination compared to conventional PCR (Bosch et al., 2011; Cliver, 1997). For instance, Butot, Putallaz, and Sánchez (2007) developed RT-PCR methods using specific primers for rapid and sensitive detection for HAV, NV and rotavirus (RV) in various types of berries and vegetables. Atmar et al. (1995) described a method for the detection of Norwalk virus and hepatitis A virus from shellfish tissues by RT-PCR with the low sample-associated interference. RT-PCR can also provide a quantitative estimate of the amount of original target present in the samples (Gibson, Heid, & Williams, 1996; Heid, Stevens, Livak, & Williams, 1996). During a RT-PCR assay, the product produced during each cycle can be quantified using an intercalating dye such as SYBR Green (via nonspecific attachment to dsDNA), or by using a fluorescent internal hybridization probe (Mackay, Arden, & Nitsche, 2002). In both cases, fluorescence is measured during each cycle, and when the amount of fluorescence exceeds the background level (threshold level), the sample is scored as positive. The number of cycles required to reach the threshold level, commonly referred to as the cycle threshold value (Ct), correlates with the amount of target in the sample prior to amplification (Heid et al., 1996).

Real-time RT-PCR is an excellent tool for detection of enteric viruses in food
products; however, one major limiting factor is the presence of inhibitory agents that interfere with reverse transcription and PCR amplification (Hyeon et al., 2010). The inhibition of amplification may originate from constituents of bacterial cells and non-target DNA, poorly controlled reaction conditions, contaminants in reagents, containers, and disposables, or from unintentional contamination during reaction preparation. The most common inhibitory components in food constituents are organic and phenolic compounds, glycogen, fats, and calcium compounds (Wilson, 1997). Sair et al. (2002b) point out that detection of viruses in lettuce samples is consistently less sensitive than in hamburger probably due to residual inhibitory compounds that continue to be relevant to plant matrices. Inhibiting substances may also persist in the extracted food samples and can in turn generate false-negative results. Internal controls for RT-PCR have been developed to determine the presence of inhibitors in a sample and ensure that samples with a negative PCR result are due to the absence of the targeted virus and not due to inhibition (Croci et al., 2008; Rodriguez et al., 2009).

2.6.3 Virus Nucleic Acid Extraction and Purification. Extraction and purification of virus nucleic acids are one of the critical steps for the detection of enteric RNA viruses from samples due to the high susceptibility of reverse transcriptase to interfering or inhibitory substances (Wilde, Eiden, & Yolken, 1990). Foods represent complex matrices from which viral nucleic acids must be extracted and purified to remove and inactivate potential inhibitors prior to being amplified and detected by RT-PCR (Fong & Lipp, 2005; Lampel, Orlandi, & Kornegay, 2000). For these purposes, a variety of methods involving multi-step elution and extraction procedures have been employed for extracting enteric viral RNA and simultaneously reducing the level of inhibitors (Butot et al., 2007; Sair et
al., 2002b). Most methods for RNA extraction from food matrices require the use of a combination of several reagents such as guanidinium isothiocyanate, cetyltrimethylammonium bromide (CTAB), phenol-chloroform sephadex, chelex, or other chemical reagents. Salt and small proteins can be effectively eliminated through the use of chelex or sephadex (De Leon et al., 1992; Straub, Pepper, & Gerba, 1994); while polysaccharides may be removed by CTAB (Boom et al., 1990). However, viral immunocapture methods, in contrast to chemical processes, are more efficient because of their specific recovery of viruses from the different inhibitory agents present in various food types (oysters, produce) (De Serres et al., 1999; Kobayashi, Natori, Takeda, & Sakae, 2004; Stals, Baert, Van Coillie, & Uyttendaele, 2012).

It is now well recognized that a range of extraction protocols have been published; many of them depend on the use of complex home-made reagents or on procedures that are not commercially available (Bianchi, Dal Vecchio, Vilarino, & Romalde, 2011). Of the “home-made” methods, one of the most widely used was proposed by Boom et al. (1990) and is based on the lysing and nuclease-inactivating agent guanidium thiocyanate along with the use of silica columns to bind and wash nucleic acids. This protocol is rapid, simple to use and efficient in removing inhibitors. Afzal and Minor (1994) have also developed an efficient method of RNA isolation without using hazardous organic chemicals and ethanol precipitation. These methods have been reported in different documents to be useful for extraction and isolation of viral RNA from vegetables and other foods (Croci, De Medici, Scalfaro, Fiore, & Toti, 2002; Jean, D’Souza, & Jaykus, 2004; Leggitt & Jaykus, 2000). Other methods, including proteinase K treatment followed by phenol-chloroform extraction and ethanol precipitation, sonication, and heat
treatment, have also been employed for viral nucleic acid extraction and purification (Albert & Schwartzbrod, 1991; Bosch, Pintó, Villena, & Abad, 1997; Chapron, Ballester, Fontaine, Frades, & Margolin, 2000; Green & Lewis, 1995; Le Guyader, Dincher, Menard, Schwartzbrod, & Pommepuy, 1994; Monpoeho et al., 2001).

The recent appearance of a number of commercial kits for the rapid isolation and purification of nucleic acids from different sources can be a valuable help offering reliable and reproducible enteric viral RNA extraction. Most of these kits are based on guanidinium lysis, and then capture of nucleic acids on a column or bead of silica. Although these commercial kits can recover viral nucleic acid while eliminating inhibitory substances efficiently, differences between each of them may be found depending on the virus and/or matrix analyzed (Bosch et al., 2011). Burgener, Candrian, and Gilgen (2003) have evaluated the detection limit and the inhibitor removal efficiency of four large-volume viral RNA extraction kits (QIAamp Viral RNA Mini Kit in combination with preconcentration by Centricon YM-100 [Centricon-QIAamp], QIAamp UltraSens Virus Kit, NucliSens Isolation Kit and NucleoSpin RNA Virus F). RT-quantitative PCR analysis has shown that both the NucliSens and Centricon-QIAamp are very sensitive, efficient methods to extract viral RNA from water samples previously concentrated. Girard, Morales-Rayas, and Jean (2013) recently have investigated four RNA extraction methods (NucliSens MiniMAG magnetic-based silica kit, bioMérieux Basic non-magnetic-based silica kit, silica-membrane-based Qiagen QIAamp MinElute Virus Spin kit and phenol-based TriReagent kit) for extracting high and low inoculation levels of murine norovirus (MNV-1) particles from simple (strawberries and lettuce) and complex (sliced turkey breast, soft-shell clams, and potato salad) food matrices. The
results indicated that of the four extraction kits, MiniMAG magnetic-based silica kit was the most effective to isolate both high and low numbers of MNV-1 particles from a wide range of foods. Other similar studies have also been conducted to compare different RNA-extraction kits for sensitive detection of hepatitis A virus in strawberry samples (Bianchi, Dal Vecchio, Vilarino, & Romalde, 2011).

The potential to recover enteric viral RNA from evidence samples has substantially increased since a variety of commercial RNA extraction kits are available. However, in order to improve the efficiency of viral RNA extraction and purification, Qiagen QIAshredder has been employed to remove inhibitory components in food samples. The QIAshredder unit consists of a unique biopolymer shredding system in a microcentrifuge spin-column format. Often, isolation of total or poly A RNA from cell or tissue lysates requires homogenization to reduce viscosity caused by high-molecular-weight cellular components and cell debris. Traditional methods use syringes and needles to cause sheer force which reduces the viscosity of the sample. This method is time-consuming, inconvenient, and hazardous (Qiagen, 2013). QIAshredder spin columns replace these homogenization methods with a fast and simple centrifugation step. In the study of improving the sensitivity and speed of enteric virus detection from food products by RT-PCR, Sair et al. (2002b) have found that the use of TRIzol with the QIAshredder homogenizer yielded the best RT-PCR detection limits.

2.7 Enteric Bacteriophages as Surrogates for Mammalian Viruses

Bacteriophages have been widely used as surrogates for mammalian viruses in medical and environmental virology applications due to several limitations related to conventional mammalian virus assays (Aranha-Creado & Brandwein, 1999). The use of
bacteriophages as surrogates for enteric viruses offers several advantages because they are non-pathogenic and methods for their recovery and enumeration are simple, rapid and inexpensive (Andreotolla et al., 2002; Davies, Yousefi, & Bavor, 2003). Virus surrogates are generally chosen due to their morphological similarity and genetic relatedness to a target virus. For example, feline calicivirus (FCV) has been considered one of the most appropriate surrogates for NoV, murine NoV (MNV) has recently been utilized as a human NoV surrogate, and poliovirus (PV) has been used as a surrogate for HAV (Green et al., 2000; Richards, 2012; Wobus, Thackray, & Virgin, 2006).

Bacteriophage MS2, morphologically similar to enteroviruses, has been evaluated previously as a model to study viral resistance to environmental stressors, disinfectants and other treatment processes (Havelaar, 1986; Havelaar, van Olphen, & Drost, 1993; WHO, 2004). In studies on the evaluation of virucidal activity of biocides, Jones, Bellamy, Alcock, and Hudson (1991) have recommended replacing human enterovirus assays with bacteriophage assays using MS2, and Figueroa, Sepulveda, Soto, and Toha (1978) have also pointed out that bacteriophage MS2 could assist in establishing viral models for human pathogens.

MS2 phage belongs to group I of the RNA coliphages within the family Leviviridae (Calender, 1988). The bacterial host for MS2 is Escherichia coli, thus, this bacteriophage is found most frequently in sewage and animal faces. MS2 is adapted to the intestinal tract and is a positive sense single-stranded RNA virus with icosahedral symmetry in the same size range at 26 nm diameters (Dawson, Paish, Staffell, Seymour, & Appleton, 2005). For virus RNA extraction study, Shulman et al. (2012) have used MS2 coliphage as an exogenous control for RT-PCR inhibition to investigate four
different RNA extraction systems (QIAamp Viral RNA Mini Kit, MagNA Pure LC2.0 Automatic extractor, KingFisher, and NucliSENS EasyMag) for extraction and co-purification of RNA from stool suspensions. The results indicated that RT-PCR for MS2 RNA was a good predictor of inhibition of enterovirus RNA extracted from stool suspensions.

2.8 The Objectives of This Study

This current study evaluated the performance of four commercial RNA extraction kits for RNA isolation and removal of inhibitors from green onion extracts prior to real-time RT-PCR detection using MS2 coliphage as an enteric virus surrogate. The kits evaluated were: Qiagen QIAamp Viral RNA Mini Kit and QIAamp UltraSens Virus Kit (Qiagen Inc., Valencia, CA), Mo Bio UltraClean Tissue & Cells RNA Isolation Kit (Mo Bio Laboratories, Inc., Carlsbad, CA), and Ambion MagMAX Viral RNA Isolation Kit (Ambion Inc., Austin, TX). The aim of this work was to compare the ability of four commercially available nucleic acid extraction kits to extract and purify MS2 coliphage RNA from (1) green onion eluates, and (2) ultracentrifuge-concentrated eluates prepared with and without Qiagen QIAshredder pre-treatment, using detection by real-time RT-PCR.
3.1 Materials

3.1.1 MS2 Coliphage. MS2 coliphage 15597-B1 (ATCC; Manassas, VA) stock was prepared by inoculating 100 μl of MS2 stock into 500 ml of a log-phase culture of *E.coli* p-Famp (ATCC 700851) host strain grown to log phase in TSBYE broth and allowing it to infect and propagate at 37 °C overnight. The host cells were removed by centrifugation at 3,000 × g for 20 min. The supernatant containing the MS2 was aliquoted into 0.5 ml volumes and frozen at -80 °C. All experiments were performed carefully using this MS2 coliphage in a bio-safety level II laboratory.

3.1.2 Eluents. To sterilely dilute the MS2 coliphage, Phosphate Buffered Saline (PBS; 0.1 M Na₂HPO₄, 0.1 M NaH₂PO₄, 0.1 M NaCl, pH 7.2) was chosen. To obtain the green onion eluates or recover MS2 coliphage from cut green onion pieces, a buffer that contained 0.75 M glycine-0.15 M NaCl (Fisher Chemical, Fair Lawn, NJ) was used. The pH of the 0.75 M glycine-0.15 M NaCl buffer was adjusted to 7.6 with 14 N NaOH using a Mettler Toledo MP 220 pH meter (Mettler-Toledo GmbH, Columbus, OH). Both eluents were autoclaved and stored at 4 °C until use.

3.1.3 Commercial RNA Extraction Kits. Four commercial RNA extraction kits to extract and purify the MS2 coliphage RNA were evaluated: Qiagen QIAamp Viral RNA Mini and QIAamp UltraSens Virus kits (Qiagen Inc., Valencia, CA), Mo Bio UltraClean Tissue & Cells RNA Isolation kit (Mo Bio Laboratories, Inc., Carlsbad, CA), and the Ambion MagMAX Viral RNA Isolation kit (Ambion Inc., Austin, TX).
3.1.4 **Green Onions.** Fresh whole green onions employed in the experiments were purchased from different local markets a day prior to the experiments. All green onions were refrigerated at 4 °C before use.

3.2 **Comparison of RNA Extraction Methods for the Detection of MS2 Coliphage from Green Onions Eluates**

3.2.1 **Green Onion Elution Preparation.** Samples of fresh whole green onions from a single lot were cut into six inch pieces by a sterile knife and a plastic cutting board. Cut green onion pieces were weighed out to 50 ± 2 g in a weigh boat (Fisher Scientific, Hanover Park, IL) with a Mettler PM600 electric balance (Mettler Instrument Corp., Hightstown, NJ) and were placed into a Whirl-pak stomacher bag (Nasco, Fort Atkinson, WI). One hundred ml of 0.75 M glycine-0.15 M NaCl buffer was added and the samples were gently treated for 1 min by a Pulsifier white 100 stomacher (Microgen Bioproducts, Ltd., UK) on low speed. The green onion eluates were recovered and distributed into 4 × 10 ml aliquots into 15 ml conical tubes (BD Falcon, Franklin lakes, NJ).

3.2.2 **MS2 Coliphage Inoculation of Green Onion Eluates.** A small aliquot of -80 °C frozen MS2 coliphage stock (approximately 10^{10} pfu/ml) was thawed at room temperature and ten-fold serially diluted in sterile PBS to obtain 10^5, 10^4, 10^3 and 10^2 pfu/ml. The 10 ml green onion eluates were inoculated with 100 µl of each dilution of MS2 coliphage stock to create final inoculation levels at 10^3, 10^2, 10^1 and 10^0 pfu/ml. As a negative control, 100 µl sterile PBS was added to a green onion eluate aliquot. For a positive control, 100 µl of 10^5 pfu/ml MS2 coliphage was inoculated directly into 10 ml sterile 0.75 M glycine-0.15 M NaCl buffer or PBS without onion matrix. After inoculation, MS2-inoculated samples/tubes were mixed thoroughly by vortexing. Aliquots of MS2-inoculated green onion eluates of each inoculation levels were
distributed into sterile, screw-capped 2 ml flat bottom tubes and stored in New Brunswick -80 °C freezer for future RNA extraction.

3.2.3 **Comparison of RNA Extraction Methods.** MS2-inoculated green onion eluates inoculated at $10^3$, $10^2$, $10^1$ and $10^0$ pfu/ml were thawed together with the green onion negative and positive controls at room temperature in a bio-safety level II cabinet. The following four different commercially available nucleic acid extraction methods were performed along with an extraction negative control (reagents only) for a total of five onion lots to extract and purify RNA from MS2 coliphage.

RNA extraction was carried out using the kit reagents [Carrier RNA (poly A), Lysis Buffer, Wash Buffer and Elution Buffer] of the Qiagen QIAamp Viral RNA Mini Kit by following the manufacturer’s instructions. Briefly, 5.6 µl of carrier RNA solution (1 µg/µl) was added to 0.56 ml lysis buffer AVL according to the volumes provided by the manufacturer’s instruction. One hundred and forty µl samples of each eluate dilution were added to 560 µl of prepared buffer AVL-carrier RNA to lyse the samples. The samples were then incubated at room temperature (15-25 °C) for 10 min. After incubation, 560 µl of ethanol (96-100 %) was added to the samples and then carefully transferred to a QIAamp Mini spin column inside a 2 ml collection tube. Spin columns were centrifuged at 6,000 x g (8,000 rpm) for 1 min, and the flow-through in the collection tube was thrown out and the spin column was placed in a new 2 ml collection tube. To wash the membrane, 500 µl of wash buffer AW1 was added in the individual spin columns and centrifuged at 6,000 x g (8,000 rpm) for 1 min, the flow-through was discarded and the spin column was placed in a new 2 ml collection tube. After the first wash, 500 µl of wash buffer AW2 was added in the individual spin columns and centrifuged at 20,000 x g
(14,000 rpm) for 3 min, flow-through was discarded and the spin column was placed in a new 2 ml collection tube. After washing, the spin column was centrifuged in the empty 2 ml collection tube for 1 min at full speed (~20,000 × g ) to dry the membrane and then transferred to a new 2 ml sterile collection tube. After that, 2 × 40 µl of elution buffer AVE was added to each membrane and samples were incubated at room temperature for 1 min. After incubation, the spin column was centrifuged at 6,000 x g (8,000 rpm) for 1 min to collect each sample’s RNA. These RNA samples were then transferred to sterile 1.5 ml microcentrifuge tubes and kept at -20 ºC prior to amplification by real-time RT-PCR assay (See Section 3.5).

For the Qiagen QIAamp UltraSens Virus Kit, RNA was extracted using the kit reagents [Carrier RNA (poly A), Proteinase K, Lysis Buffer, Resuspension Buffer, Binding Buffer, Wash Buffer and Elution Buffer] by following the manufacturer’s instructions. Briefly, 5.6 µl of carrier RNA solution (1 µg/µl) was added into each tube lid, followed by addition of 0.8 ml of lysis buffer AC on top of 1 ml samples of each eluate dilution mixed thoroughly by closing the lid, first inverting the sample tubes 3 times and then vortexing for 10 sec to lyse the samples. The samples were then incubated at room temperature (15-25 ºC) for 10 min. After incubation, the samples were centrifuged at 1,200 × g for 3 min and the supernatant was completely removed and discarded. Then, 300 µl of freshly made warmed resuspend buffer AR (60 ºC) containing 20 µl of proteinase K solution was then added to the samples and incubated at 40 ºC for 10 min in an AccuBlock digital dry bath (Labnet International, Inc., Model D1200, Woodbridge, NJ). After incubation, 300 µl binding buffer AB was added into the samples and then carefully transferred to a QIAamp spin column inside a 2 ml collection tube.
Spin columns were centrifuged at 3,000-5,000 x g for 1 min. Flow-through in the collection tube was discarded and the spin column was placed in a new 2 ml collection tube. To wash the membrane, 500 µl of wash buffer AW1 was added in the individual spin columns and centrifuged at 6,000 x g for 1 min, flow-through was thrown out and the spin column was placed in a new 2 ml collection tube. After first wash, 500 µl of wash buffer AW2 was added in the individual spin columns and centrifuged at full speed (~20,000 x g) for 3 min, flow-through was discarded and the spin column was placed in a new 2 ml collection tube. After washing, the spin column was centrifuged in the empty 2 ml collection tube for 1 min at full speed (~20,000 x g) to dry the membrane and then transferred to a new 2 ml sterile collection tube. After that, 2 x 30 µl of elution buffer AVE was carefully applied to each membrane and the spin column was centrifuged at 6,000 x g for 1 min to elute sample’s RNA. The extracted RNA samples were then transferred to sterile 1.5 ml microcentrifuge tubes and stored at -20 °C prior to real-time RT-PCR amplification.

For the Mo Bio UltraClean Tissue & Cells RNA Isolation Kit, RNA extraction was performed using the kit reagents [β-mercaptoethanol (βME), Lysis Buffer, Binding Buffer, Wash Buffer and Elution Buffer] by following the manufacturer’s instructions. For all samples to be processed, 10 µl of β-mercaptoethanol was added to every 1 ml of solution TR1 and 300 µl of this prepared lysis solution TR1 was added to 300 µl samples of each eluate dilution in a sterile 2ml collection tube. After vortexing for 2 min, 1 volume (300 µl) of binding solution TR2 was added into the samples and then carefully transferred to a spin column inside a 2 ml collection tube. Spin filters were centrifuged for 1 min at ≥ 10,000 x g, filtrate in the collection tube was thrown out and the spin filter
was placed in a new 2 ml collection tube. To wash the membrane, 500 µl of wash solution TR3 was added in the individual spin filters and centrifuged for 1 min at ≥ 10,000 × g, filtrate was discarded and the spin filter was placed in a new 2 ml collection tube. After washing with solution TR3, 2 × 500 µl of wash solution TR4 was added in the individual spin filters and centrifuged for 1 min at ≥ 10,000 × g, filter was discarded and the spin filter was placed in a new 2 ml collection tube. After washing, the spin filter was centrifuged in the empty 2 ml collection tube for 2 min at 13,000 × g to dry the membrane and then transferred to a new 2 ml sterile collection tube. Elution buffer TR5 (50 µl) was added onto each membrane and samples were incubated at room temperature for 1 min followed by centrifugation for 1 min at ≥ 10,000 × g to recover the RNA. RNA was kept at -20 °C prior to real-time RT-PCR amplification.

For the Ambion MagMAX Viral RNA Isolation Kit, RNA was extracted using the kit reagents (Carrier RNA, Lysis/Binding Buffer, Wash Solution and Elution Buffer) and RNA Binding Beads by following the manufacturer’s instructions. Briefly, 2 µl of carrier RNA was added to 400 µl of lysis/binding solution concentrate, followed by addition of 400 µl of isopropanol (100 %) according to the volumes provided by the manufacturer’s instruction. Bead mix was freshly prepared by combining 10 µl RNA Binding Beads with 10 µl lysis/binding enhancer per reaction and kept on ice until needed. Four hundred µl samples of each eluate dilution were added to 802 µl of prepared lysis/binding solution (carrier RNA and isopropanol added) in a processing tube. Twenty µl of bead mix was added to each sample and the samples were gently shaken for 4 min on a vortex mixer to fully lyse viruses and bind RNA to the RNA Binding Beads. The RNA Binding Beads were captured by placing the processing tubes into a magnetic stand for at least 3 min and
the supernatant was carefully aspirated and thrown out without disturbing the beads after capture. The processing tubes were removed from the magnetic stand and the beads were washed by the addition of $2 \times 300 \, \mu l$ Wash Solution 1 (isopropanol added). After vortexing at moderate speed for 30 sec, the RNA Binding Beads were captured by placing the processing tubes to the magnetic stand for 3-5 min and the supernatant was carefully aspirated and discarded without disturbing the beads. Then, the processing tubes were moved off the magnetic stand. After washing with Wash Solution 1, $2 \times 450 \, \mu l$ Wash Solution 2 (ethanol added) was added to each sample. After vortexing at moderate speed for 30 sec, the RNA Binding Beads were captured as in the previous wash. The supernatant was then carefully aspirated and discarded without disturbing the beads and the processing tubes were removed from the magnetic stand. After washing, the beads were dried by leaving the tubes open on the bench for 2 min to remove the residual solution. To elute the RNA, 50 $\mu l$ Elution Buffer at room temperature was added to each sample and the tubes were shaken vigorously for 4 min. The RNA Binding Beads were captured as in the previous steps. The supernatant, which contained the purified RNA, was then transferred to a nuclease-free 1.5 ml microcentrifuge tube and stored at -20 °C prior to real-time RT-PCR amplification.

3.3 Comparison of RNA Extraction Methods for the Detection of MS2 Coliphage from Concentrated Green Onions Eluates

3.3.1 Green Onion Elution Preparation. Samples of fresh whole green onions from a single lot were cut into six inch pieces by a sterile knife and a plastic cutting board. Cut green onion pieces were weighed out into $8 \times 50 \pm 2 \, g$ in weigh boats and were placed into Whirl-pak stomacher bags. Each sample bag was filled with 55 ml of 0.75 M glycine-0.15 M NaCl (pH 7.6) buffer and sealed tightly. The samples were placed in a
holder and shaken at 150 rpm at room temperature for 15 min in an Innova 4430 incubator shaker (New Brunswick Scientific, Edison, NJ). Then samples were removed from the shaker and the green onion eluates from each sample bag were completely recovered to a clean 100 ml F40L-8 × 100 rotor tube (ThermoFisher Scientific, Asheville, NC).

3.3.2 Green Onion Eluate Inoculation and Concentration. Green onion eluates were centrifuged at 12,000 × g (11,000 rpm) for 30 min at 4 ºC using a Sorvall WX Ultra 80 Series Centrifuge (ThermoFisher Scientific, Asheville, NC) to clear the samples of large particulates. The supernatant was carefully recovered without disturbing the green onion particulates and transferred to a clean 100 ml F40L-8 × 100 rotor tube. A small aliquot of -80 ºC frozen MS2 coliphage stock (approximately 10¹⁰ pfu/ml) was ten-fold serially diluted in sterile PBS to get the dilution level of 10⁴ pfu/ml. Each 50 ml (approximately ultracentrifuge-concentrated green onion eluate sample was inoculated with 200 µl of 10⁴ pfu/ml MS2 coliphage stock to obtain a final inoculation level of 40 pfu/ml (2000 pfu/50 g onion sample). Then the tubes were mixed thoroughly using the vortex mixer.

3.3.3 MS2 Coliphage Concentration in Eluates. To concentrate MS2 coliphage in eluates prior to RNA extraction, samples/rotor tubes were balanced with additional glycine-NaCl buffer with the total volume to be at least 50 ml. The tubes were then transferred to a Fiberlite F40L-8 × 100 rotor and ultracentrifuged at 170,000 × g (37,000 rpm) for 60 min at 4 ºC using a Sorvall WX Ultra 80 Series Centrifuge. The supernatant was carefully poured off and the residual eluates were completely removed after letting the tubes rest an additional 4-5 min without disturbing the pellet. The MS2 coliphage pellet was resuspended with 300 µl of sterile PBS and homogenized by
votexing/pipetting. The eluate-PBS mixture containing MS2 coliphage particles from each 100 ml F40L-8 × 100 rotor tube was carefully transferred to sterile, screw-capped 2 ml flat bottom tubes and stored in a -80 °C freezer for future extraction.

3.3.4 **Comparison of RNA Extraction Methods (with and without Qiagen QIAshredder Pre-treatment).** MS2-inoculated green onion ultracentrifuge-concentrated eluates inoculated at 40 pfu/ml (2000 pfu/50 g) were thawed at room temperature in a bio-safety level II cabinet. The following modified four different commercially available nucleic acid extraction methods were performed together with an extraction negative (reagents only) and positive controls to extract and purify RNA from MS2 coliphage, with and without Qiagen QIAshredder pre-treatment.

For the Qiagen QIAamp Viral RNA Mini Kit, RNA was extracted by following the modified manufacturer’s instructions. For each reaction, 5.6 µl of carrier RNA solution (1 µg/µl) was added to 0.56 ml lysis buffer AVL according to the volumes provided by the manufacturer’s instruction. To the 300 µl sample, 1.12 ml of prepared buffer AVL-carrier RNA was added to lyse the samples. Then the samples were incubated at room temperature (15-25 °C) for 10 min. After incubation, samples were put through a Qiagen QIAshredder and centrifuged at full speed (~20,000 × g) for 1 min and filtrates were recovered to a clean 15 ml tube. For both the QIAshredder pre-treated and non pre-treated samples, an additional 1.12 ml of ethanol (96-100 %) was added. The extraction procedure was continued as previously described for MS2 coliphage RNA extraction from green onions eluates (See Section 3.2.3). As an extraction positive control, 200 µl of 10⁴ pfu/ml MS2 coliphage was added directly into 1.12 ml buffer AVL-carrier RNA and extracted similarly.
For the Qiagen QIAamp UltraSens Virus Kit, RNA extraction was carried out by following the modified manufacturer’s instructions. An additional 700 µl sterile PBS was added to each 300 µl sample to create a 1 ml sample solution. For each 1 ml sample, 5.6 µl of carrier RNA solution (1 µg/µl) was added into each tube lid, followed by addition of 0.8 ml of lysis buffer on top of sample mixed thoroughly by closing the lid, first inverting the sample tubes 3 times and then vortexing for 10 sec to lyse the samples. The samples were then incubated at room temperature (15-25 ºC) for 10 min. After incubation, samples were put through a Qiagen QIAshredder and centrifuged at full speed (~20,000 × g) for 1 min and filtrates were recovered to a clean 2 ml tube. For both the QIAshredder pre-treated and non pre-treated samples, the extraction procedure was continued as previously described for MS2 coliphage RNA extraction from green onions eluates (See Section 3.2.3). As an extraction positive control, 800 µl of PBS was added to 200 µl of 10⁴ pfu/ml MS2 coliphage to create a 1 ml sample solution, then 5.6 µl of carrier RNA solution (1 µg/µl) was added into the tube lid, followed by the addition of 0.8 ml of lysis buffer on top of sample.

For the Mo Bio UltraClean Tissue & Cells RNA Isolation Kit, RNA was extracted by following the modified manufacturer’s instructions. For all samples to be processed, 10 µl of β-mercaptoethanol was added to every 1 ml of the solution TR1 and 300 µl of this prepared lysis solution TR1 was added to each 300 µl sample in a sterile 2 ml collection tube. After vortexing for 2 min, the entire 600 µl sample was put through a Qiagen QIAshredder and centrifuged at full speed (~20,000 × g) for 1 min and filtrates were collected in a clean 2 ml tube. For both the QIAshredder pre-treated and non pre-treated samples, 1 volume (300 µl) of solution TR2 was added and the extraction
procedure was continued as previously described for MS2 coliphage RNA extraction from green onions eluates (See Section 3.2.3). As an extraction positive control, 200 µl of $10^4$ pfu/ml MS2 coliphage was added into 300 µl solution TR1.

For the Ambion MagMAX Viral RNA Isolation Kit, RNA extraction was performed by following the modified manufacturer’s instructions. For each reaction, 2 µl of carrier RNA was added to 400 µl of lysis/binding solution concentrate, followed by addition of 400 µl of isopropanol (100 %) according to the volumes provided by the manufacturer’s instruction. Bead mix was freshly prepared by combining 10 µl RNA Binding Beads with 10 µl lysis/binding enhancer per reaction and kept on ice until needed. To each 300 µl sample, 802 µl of prepared lysis/binding solution (carrier RNA and isopropanol added) was added. After gentle vortexing for 2 min, the samples were put through a Qiagen QIAshredder and centrifuged at full speed (~20,000 × g) for 1 min and filtrates were transferred to a clean processing tube. For both the QIAshredder pre-treated and non pre-treated samples, 20 µl of bead mix was added to each sample and the samples were gently shaken for 4 min on a vortex mixer to fully lyse viruses and bind RNA to the RNA Binding Beads. The extraction procedure was then continued the same as previously described for MS2 coliphage RNA extraction from green onions eluates (See Section 3.2.3). As an extraction positive control, 200 µl of $10^4$ pfu/ml MS2 coliphage was added into 802 µl prepared lysis/binding solution.

3.4 Comparison of Qiagen QIAamp Mini and MagMAX Viral RNA Extraction Methods for the Detection of MS2 Coliphage from Green Onions

3.4.1 MS2 Coliphage Inoculation on Green Onions. Samples of fresh whole green onions from a single lot were cut into six inch pieces by a sterile knife and a plastic cutting board. Cut green onion pieces were weighed out into $8 \times 50 \pm 2$ g in a large weigh
boat with a Mettler PM600 electric balance. A small aliquot of -80 °C frozen MS2 coliphage stock (approximately $10^{10}$ pfu/ml) was thawed at room temperature and diluted by 5-fold in sterile PBS, followed by ten-fold serially diluted to get the first inoculation level of 1000 pfu/500 µl. Then, the above culture (1000 pfu/500 µl) was further diluted 4-fold to create the second inoculation level of 250 pfu/500 µl. Four weigh boats of green onions, each weigh boat containing 50 ± 2 g green onion pieces, were inoculated with 500 µl of each dilution levels of MS2 coliphage stock with a micro pipette by gently spreading drops of the stock over as many pieces as possible to get the two inoculation levels of 20 pfu/g and 5 pfu/g. The inoculated green onion samples were left in a biosafety level II cabinet and air-dried for 1 hr.

3.4.2 Recovery of MS2 Coliphage from Inoculated Green Onion. Each 50 ± 2 g inoculated green onion sample was carefully transferred to a Whirl-Pak stomacher bag filled with 50-55 ml of 0.75 M glycine-0.15 M NaCl (pH 7.6) buffer and sealed tightly. The samples were placed in a holder and shaken at 150 rpm at room temperature for 15 min in an Innova 4430 incubator shaker. Then samples were removed from the shaker and the green onion eluates containing MS2 coliphage from each sample bag were completely recovered to a clean 100 ml F40L-8 × 100 rotor tube.

3.4.3 MS2 Coliphage Concentration in Eluates. To concentrate MS2 coliphage in green onion eluates, eight 100 ml F40L-8 × 100 rotor tubes containing two inoculation levels of MS2 coliphage were balanced with additional glycine-NaCl buffer with the total volume to be at least 50 ml. The tubes were then transferred to a Fiberlite F40L-8 × 100 rotor and centrifuged at 12,000 × g (11,000 rpm) for 30 min at 4 °C using a Sorvall WX Ultra 80 Series Centrifuge to remove large particulates. The supernatant was carefully
recovered without disturbing the green onion particulates to a clean 100 ml F40L-8 × 100 rotor tube. Prior to ultracentrifugation, additional glycine-NaCl buffer was added to samples to balance rotor tubes with the total sample volume of at least 50 ml. The tubes were then transferred to a Fiberlite F40L-8 × 100 rotor and ultracentrifuged at 170,000 × g (37,000 rpm) for 60 min at 4 °C using a Sorvall WX Ultra 80 Series Centrifuge. The supernatant was carefully poured off and the residual eluates were completely removed after letting the tubes rest an additional 4-5 min without disturbing the pellet. After concentration, the MS2 coliphage pellet was resuspended with 300 µl of sterile PBS and homogenized by vortexing/pipetting. The eluate-PBS mixture containing MS2 coliphage particles from each 100 ml F40L-8 × 100 rotor tube was carefully transferred to sterile, screw-capped 2 ml flat bottom tubes and stored in a -80 °C freezer for future extraction.

3.4.4 Comparison of QIAamp and MagMAX Viral RNA Extraction Methods (with and without Qiagen QIAshredder Pre-treatment). MS2 coliphage inoculated at 20 and 5 pfu/g on green onions was eluted and ultracentrifuge-concentrated prior to RNA extraction. Samples that were thawed at room temperature in a bio-safety level II cabinet were extracted using the Qiagen QIAamp Viral RNA Mini Kit and Ambion MagMAX Viral RNA Isolation Kit as previously described along with extraction negative (reagents only) and positive controls, with and without Qiagen QIAshredder pre-treatment (See Section 3.3.4).

3.5 MS2 Coliphage and Internal Control RNA Quantification by Real-time RT-PCR Assay

A quantitative real-time Taqman probe-based RT-PCR assay was carried out to amplify the MS2 coliphage RNA together with a non-competitive internal amplification control for evaluation of inhibition. Each reaction had a total final volume of 25 µl which
contained 5 µl of extracted RNA template and 20 µl of PCR reagents in a master mix. Five µl of Quantitect probe PCR mix (Qiagen), 1 µl of each forward and reverse primer, 0.5 µl of probe and 3 µl of MgCl₂ were mixed together to prepare the master mix. For MS2 detection, a FAM probe-based RT-PCR assay targeting a sequence for the MS2 assembly protein was utilized (O’Connell et al., 1996). For the exogenous internal control, a Texas Red probe-based internal amplification control (IAC) assay was employed, the primers, probe, and synthetic RNA template were supplied by FDA. A list of all the reagents used to make the master mix is shown in Table 3.1. Real-time RT-PCR reaction was performed in a Roche LightCycler 480 (Applied Science, Indianapolis, IN) under the following cycling conditions: initiation (96 ºC for 15 min), followed by 45 cycles of amplification (96 ºC for 10 sec, 60 ºC for 30 sec). All experiments were identical in concentration of primers and probes and also in temperature conditions. A negative no template (water) control was always run in each experiment to test for any false positive or contamination in the sample. The sequence of primers and probe used to detect MS2 coliphage is given in Table 3.2 and all were manufactured by Integrated DNA Technologies, Inc., Coralville, IA.

For any negative PCR results, the samples were diluted 10-fold and 100-fold with nucleic acid free water and amplified by real-time RT-PCR assay in duplicate to determine if detection was possible with dilution of the template.

### 3.6 Statistical Analysis

The results of the threshold cycles (Cₜ) were recorded for each reaction and were compared with each RNA extraction method to evaluate their ability to extract and purify MS2 coliphage RNA from the onion samples. All statistical analyses were carried out by
using the software originally equipped in the Roche LightCycler 480 and Microsoft Excel 2007 (Microsoft, Redmond, WA).
Table 3.1. Composition of PCR reaction

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Stock</th>
<th>Final Concentration</th>
<th>(For single reaction) Final Volume/25 µl reaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Quantitect Probe mix</td>
<td>5X</td>
<td>1X</td>
<td>5 µl</td>
</tr>
<tr>
<td>MgCl₂</td>
<td>25 mM</td>
<td>3 mM</td>
<td>3 µl</td>
</tr>
<tr>
<td>SuperaseIn</td>
<td>-</td>
<td>-</td>
<td>0.25 µl</td>
</tr>
<tr>
<td>dNTP Mix</td>
<td>-</td>
<td>-</td>
<td>1 µl</td>
</tr>
<tr>
<td>MS2-F primer</td>
<td>10 µM</td>
<td>0.4 µM</td>
<td>1 µl</td>
</tr>
<tr>
<td>MS2-R primer</td>
<td>10 µM</td>
<td>0.4 µM</td>
<td>1 µl</td>
</tr>
<tr>
<td>MS2-Probe</td>
<td>10 µM</td>
<td>0.2 µM</td>
<td>0.5 µl</td>
</tr>
<tr>
<td>IC-F primer</td>
<td>10 µM</td>
<td>0.075 µM</td>
<td>0.1875 µl</td>
</tr>
<tr>
<td>IC-R primer</td>
<td>10 µM</td>
<td>0.075 µM</td>
<td>0.1875 µl</td>
</tr>
<tr>
<td>IC-Probe</td>
<td>10 µM</td>
<td>0.15 µM</td>
<td>0.375 µl</td>
</tr>
<tr>
<td>IC RNA</td>
<td>-</td>
<td>-</td>
<td>0.2 µl</td>
</tr>
<tr>
<td>Water</td>
<td>-</td>
<td>-</td>
<td>6.05 µl</td>
</tr>
<tr>
<td>Enzyme Mix</td>
<td>-</td>
<td>-</td>
<td>1.25 µl</td>
</tr>
<tr>
<td>RNA Template</td>
<td>-</td>
<td>-</td>
<td>5 µl</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>-</td>
<td>-</td>
<td>25 µl</td>
</tr>
</tbody>
</table>

Table 3.2. Sequences of primers and probes specific to genome of MS2 and Internal Control RNA in real-time RT-PCR assay

<table>
<thead>
<tr>
<th>Primer/Probe</th>
<th>Sequences (5’-3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MS2 Forward</td>
<td>GTC GCG GTA ATT GGC GC</td>
</tr>
<tr>
<td>MS2 Reverse</td>
<td>GGC CAC GTG TTT TGA TCG A</td>
</tr>
<tr>
<td>MS2 Probe</td>
<td>FAM- AGG CGC TCC GCT ACC TTG CCC T-IBQ</td>
</tr>
</tbody>
</table>
CHAPTER 4
RESULTS AND DISCUSSION

Real-time reverse transcriptase-polymerase chain reaction (RT-PCR) has been widely used in the field of food virology for detection enteric viruses from contaminated foods such as fresh produce due to its high speed and sensitivity. Unfortunately, viral loads found in foods are often at low contamination levels and co-extracted substances from foods may inhibit detection. Additionally, sample preparation methods appropriate for foods are not well developed and will vary based on the type of food. Therefore, sample preparation procedures for fresh produce are required evaluation to determine the techniques which allow the best recovery of enteric viruses without inhibiting substances carried over into the PCR reactions.

4.1 Comparison of RNA Extraction Methods for the Detection of MS2 Coliphage from Green Onions Eluates

4.1.1 Comparison of RNA Extraction Kits for Detection of MS2 RNA. Green onion eluates containing $10^3$-$10^0$ pfu/ml MS2 coliphage were subjected to four different commercial RNA extraction methods and then analyzed by the standard real-time RT-PCR assay. The overall concentration factor (input volume/elution volume) for each kit tested was 1.75X for QIAamp Mini kit, 16.7X for QIAamp UltraSens kit, 6X for Mo Bio Ultraclean kit, and 8X for Ambion MagMAX kit. Detection results obtained at inoculation levels of $10^3$ pfu/ml (Table 4.1) and $10^2$ pfu/ml (Table 4.2) show that the MS2 RNA extracted by QIAamp Mini kit failed to be detected in onion lot C, whereas the other kits allowed detection in replicate amplifications at $10^3$ pfu/ml, and single amplifications for QIAamp Mini and MagMAX in onion lot B, for Mo Bio Ultraclean in
onion lots D and E at $10^2$ pfu/ml. For the inoculation level of $10^1$ pfu/ml (Table 4.3), the QIAamp UltraSens only allowed detection of MS2 in onion lot A with 1 of 2 positive amplifications, while Mo Bio Ultraclean and MagMAX allowed MS2 detection in onion lots A and C with 1 of 2 positive amplifications. QIAamp Mini showed the best detection with both onion lots A and D detected in replicate amplifications. The results shown in Table 4.4 indicate that all kits were capable of detecting two onion lots at the lowest inoculation level of $10^0$ pfu/ml. Mo Bio Ultraclean and MagMAX detected only one of duplicate amplifications for the two positive lots, while QIAamp Mini and QIAamp UltraSens had better detection of one lot in duplicate amplifications.

The number of MS2 positives from five onion eluate lots each inoculated at $10^3$, $10^2$, $10^1$ and $10^0$ pfu/ml is shown in Table 4.5. The results indicated that the QIAamp Mini kit was somewhat problematic with only 4/5 lots detected at $10^3$-$10^2$ pfu/ml, whereas the other kits were positive for 5/5 lots at the $10^3$ pfu/ml inoculation level. The MagMAX kit did have reduced detection at the $10^2$ pfu/ml level with only 4/5 samples positive. All kits were capable of detecting 2/5 at the inoculation level of $10^1$ pfu/ml except the QIAamp UltraSens kit with only 1/5 samples positive. In general, all kits were able to detect samples at the lowest inoculation level of $10^0$ pfu/ml with 2/5 lots having positive detection.

The commercial viral RNA extraction methods compared in this study are based on using silica membrane spin column (QIAamp Mini, QIAamp UltraSens, Mo Bio Ultraclean), and microspherical paramagnetic beads (MagMAX) for RNA extraction. Recent research conducted by Girard, Morales-Rayas, and Jean (2013) has shown similar results for the detection of murine norovirus (MNV-1) in ready-to-eat foods. With real-
time RT-PCR, the silica membrane spin-column-based method (Qiagen QIAamp MinElute Virus Spin kit) obtained a detection viral load level at 0.02 pfu/ml, 1.21 pfu/ml, 2.12 pfu/ml for lettuce, soft-shell clams, and sliced turkey breast, respectively with Pulsifier pre-treatment. When prior treated by repetitive pipetting, this kit achieved detection at 0.86 pfu/ml, 0.76 pfu/ml, and 0.89 pfu/ml for MNV-1 on lettuce, sliced turkey breast, and strawberries, respectively. On the other hand, Kingsley and Richards (2001) developed a virus extraction procedure, involving the use of a pH 9.5 glycine buffer, polyethylene glycol (PEG) precipitation, Tri-reagent extraction and magnetic poly(dT) beads capture, detecting HAV as low as 0.015 PFU and 22.4 RT-PCR_{50} units for Norwalk virus in shellfish with one-step RT-PCR.

4.1.2 Comparison of Inhibition Removal after RNA Extraction by Commercial Extraction Kit. The four commercially available nucleic acid extraction kits were evaluated for eliminating potential PCR inhibitors that might be present in the green onion eluates. The degree of PCR inhibitory effect was assessed by the shift in cycle threshold (Ct) values of internal amplification control (IAC) between the uninoculated green onion eluates and no matrix samples. Results showing the degree of PCR inhibition for each extraction procedure in five onion lots are presented in Table 4.6. The internal amplification control (IAC) assay indicated that the greatest inhibition resulted from using the magnetic bead-based MagMAX kit with 3/5 lots at an average of 1.5 cycle shift, followed by the silica member spin-column-based kits. The QIAamp UltraSens had 2/5 lots inhibited at an average of 2.5 cycle shift, Mo Bio Ultracean had 2/5 lots inhibited at an average of 0.5 cycle shift, and QIAamp Mini had only 1/5 lots inhibited, showing a 1.7
cycle shift. However, it appeared that the degree of PCR inhibitory effect of each lot was not consistent across lots with the most inhibition overall from green onion lot D.

The plausible reason behind the better detection from silica member spin-column-based kits may be that when samples go through rigorous extraction processes using strong reagents, viral RNA bound to the silica membrane is retained while compounds which would inhibit downstream enzymatic reactions are removed during centrifugation under proper salt and pH conditions. Whereas, the high inhibition of magnetic bead-based MagMAX kit could have been due to the positively charged beads which are non-specific, and consequently bind to residual inhibitors from onion matrix when capturing the viruses. In contrast to our results showing that the greatest inhibition of real-time RT-PCR resulted from using magnetic bead-based kit (MagMAX), previous research by Shulman et al. (2012) using MS2 as an exogenous control to evaluate four different systems for extraction of RNA from stool suspensions, however, has found out that RNA extracted via magnetic bead-based systems contained fewer inhibitors than column-based systems.
Table 4.1. Comparison of four RNA extraction kits for detection of MS2 coliphage at inoculation level of $10^3$ pfu/ml by real-time RT-PCR

<table>
<thead>
<tr>
<th>Onion Lots&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Extraction Kits&lt;sup&gt;b&lt;/sup&gt;</th>
<th>QIAamp Mini&lt;sup&gt;c&lt;/sup&gt;</th>
<th>QIAamp UltraSens&lt;sup&gt;c&lt;/sup&gt;</th>
<th>Mo Bio Ultraclean&lt;sup&gt;c&lt;/sup&gt;</th>
<th>MagMAX&lt;sup&gt;c&lt;/sup&gt;</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>C&lt;sub&gt;t&lt;/sub&gt;</td>
<td>SD&lt;sup&gt;d&lt;/sup&gt;</td>
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<td>SD</td>
<td>C&lt;sub&gt;t&lt;/sub&gt;</td>
</tr>
<tr>
<td>A</td>
<td>32.69</td>
<td>0.06</td>
<td>32.03</td>
<td>0.02</td>
<td>36.76</td>
</tr>
<tr>
<td>B</td>
<td>39.87</td>
<td>1.39</td>
<td>36.62</td>
<td>0.14</td>
<td>36.67</td>
</tr>
<tr>
<td>C</td>
<td>41.20*</td>
<td></td>
<td>36.13</td>
<td>0.54</td>
<td>36.78</td>
</tr>
<tr>
<td>D</td>
<td>35.70</td>
<td>0.08</td>
<td>35.65</td>
<td>0.19</td>
<td>36.36</td>
</tr>
<tr>
<td>E</td>
<td>31.71</td>
<td>0.02</td>
<td>35.67</td>
<td>0.19</td>
<td>36.91</td>
</tr>
</tbody>
</table>

<sup>a</sup> The four extraction procedures were conducted separately with the same lot of fresh green onions.

<sup>b</sup> The standard maximum volumes that could be extracted: 140 μl for QIAamp Mini; 1 ml for QIAamp UltraSens; 300 μl for Mo Bio Ultraclean; 400 μl for MagMAX.

<sup>c</sup> Cycle threshold (C<sub>t</sub>) values corresponded to MS2 RNA detected were obtained per duplicate.

<sup>d</sup> Standard deviation

ND=Not detected

*=1/2<sup>+</sup> amplifications
Table 4.2. Comparison of four RNA extraction kits for detection of MS2 coliphage at inoculation level of $10^2$ pfu/ml by real-time RT-PCR

<table>
<thead>
<tr>
<th>Extraction Kits&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Onion Lots&lt;sup&gt;a&lt;/sup&gt;</th>
<th>QIAamp Mini&lt;sup&gt;c&lt;/sup&gt;</th>
<th>QIAamp UltraSens&lt;sup&gt;c&lt;/sup&gt;</th>
<th>Mo Bio Ultraclean&lt;sup&gt;c&lt;/sup&gt;</th>
<th>MagMAX&lt;sup&gt;c&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>C&lt;sub&gt;t&lt;/sub&gt;</td>
<td>SD&lt;sup&gt;d&lt;/sup&gt;</td>
<td>C&lt;sub&gt;t&lt;/sub&gt;</td>
<td>SD</td>
<td>C&lt;sub&gt;t&lt;/sub&gt;</td>
</tr>
<tr>
<td>A</td>
<td>35.92</td>
<td>0.56</td>
<td>38.53</td>
<td>0.32</td>
<td>39.42</td>
</tr>
<tr>
<td>B</td>
<td>40.10*</td>
<td></td>
<td>38.92</td>
<td>0.47</td>
<td>40.11</td>
</tr>
<tr>
<td>C</td>
<td>ND</td>
<td></td>
<td>38.12</td>
<td>0.34</td>
<td>38.84</td>
</tr>
<tr>
<td>D</td>
<td>38.27</td>
<td>0.39</td>
<td>38.91</td>
<td>0.27</td>
<td>40.18*</td>
</tr>
<tr>
<td>E</td>
<td>35.97</td>
<td>0.23</td>
<td>36.04</td>
<td>0.37</td>
<td>38.72*</td>
</tr>
</tbody>
</table>

<sup>a</sup> The four extraction procedures were conducted separately with the same lot of fresh green onions.

<sup>b</sup> The standard maximum volumes that could be extracted: 140 μl for QIAamp Mini; 1 ml for QIAamp UltraSens; 300 μl for Mo Bio Ultraclean; 400 μl for MagMAX.

<sup>c</sup> Cycle threshold (C<sub>t</sub>) values corresponded to MS2 RNA detected were obtained per duplicate.

<sup>d</sup> Standard deviation

ND=Not detected

*=1/2<sup>+</sup> amplifications
Table 4.3. Comparison of four RNA extraction kits for detection of MS2 coliphage at inoculation level of $10^1$ pfu/ml by real-time RT-PCR

<table>
<thead>
<tr>
<th>Onion Lots&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Extraction Kits&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Extraction Kits&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Extraction Kits&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Extraction Kits&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>QIAamp Mini</td>
<td>QIAamp UltraSens</td>
<td>Mo Bio Ultraclean</td>
<td>MagMAX</td>
</tr>
<tr>
<td></td>
<td>$C_t^c$ SD&lt;sup&gt;d&lt;/sup&gt;</td>
<td>$C_t$ SD</td>
<td>$C_t$ SD</td>
<td>$C_t$ SD</td>
</tr>
<tr>
<td>A</td>
<td>40.37 0.11</td>
<td>40.57*</td>
<td>40.25*</td>
<td>39.96*</td>
</tr>
<tr>
<td>B</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>C</td>
<td>ND</td>
<td>ND</td>
<td>40.24*</td>
<td>39.71*</td>
</tr>
<tr>
<td>D</td>
<td>39.72 0.43</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>E</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

<sup>a</sup> The four extraction procedures were conducted separately with the same lot of fresh green onions.

<sup>b</sup> The standard maximum volumes that could be extracted: 140 μl for QIAamp Mini; 1 ml for QIAamp UltraSens; 300 μl for Mo Bio Ultraclean; 400 μl for MagMAX.

<sup>c</sup> Cycle threshold ($C_t$) values corresponded to MS2 RNA detected were obtained per duplicate.

<sup>d</sup> Standard deviation

ND=Not detected

*=1/2 + amplifications
Table 4.4. Comparison of four RNA extraction kits for detection of MS2 coliphage at inoculation level of $10^0$ pfu/ml by real-time RT-PCR

<table>
<thead>
<tr>
<th>Onion Lots&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Extraction Kits&lt;sup&gt;b&lt;/sup&gt;</th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>QIAamp Mini&lt;sup&gt;c&lt;/sup&gt;</td>
<td>QIAamp UltraSens</td>
<td>Mo Bio Ultraclean</td>
<td>MagMAX</td>
<td></td>
</tr>
<tr>
<td></td>
<td>$C_t$</td>
<td>SD&lt;sup&gt;d&lt;/sup&gt;</td>
<td>$C_t$</td>
<td>SD</td>
<td>$C_t$</td>
</tr>
<tr>
<td>A</td>
<td>ND</td>
<td></td>
<td>40.22*</td>
<td></td>
<td>ND</td>
</tr>
<tr>
<td>B</td>
<td>ND</td>
<td></td>
<td>ND</td>
<td></td>
<td>ND</td>
</tr>
<tr>
<td>C</td>
<td>ND</td>
<td></td>
<td>ND</td>
<td>40.03*</td>
<td>ND</td>
</tr>
<tr>
<td>D</td>
<td>40.16*</td>
<td>0.14</td>
<td>ND</td>
<td>40.46*</td>
<td>ND</td>
</tr>
<tr>
<td>E</td>
<td>39.00</td>
<td>0.14</td>
<td>39.57</td>
<td>0.25</td>
<td>ND</td>
</tr>
</tbody>
</table>

<sup>a</sup> The four extraction procedures were conducted separately with the same lot of fresh green onions.

<sup>b</sup> The standard maximum volumes that could be extracted: 140 µl for QIAamp Mini; 1 ml for QIAamp UltraSens; 300 µl for Mo Bio Ultraclean; 400 µl for MagMAX.

<sup>c</sup> Cycle threshold ($C_t$) values corresponded to MS2 RNA detected were obtained per duplicate.

<sup>d</sup> Standard deviation

ND=Not detected

*=1/2<sup>+</sup> amplifications
Table 4.5. Limit of four RNA extraction kits for detection of MS2 coliphage at inoculation levels from $10^3$ to $10^0$ pfu/ml with respect to number of MS2 positive samples/total samples

| MS2 coliphage (pfu/ml) | No.of MS2 positives/No.of total samples
<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>QIAamp Mini</td>
</tr>
<tr>
<td>$10^3$</td>
<td>4/5</td>
</tr>
<tr>
<td>$10^2$</td>
<td>4/5</td>
</tr>
<tr>
<td>$10^1$</td>
<td>2/5</td>
</tr>
<tr>
<td>$10^0$</td>
<td>2/5</td>
</tr>
</tbody>
</table>

*a Cutoff $C_t$ >41

Table 4.6. Internal Amplification Control (IAC) assay results for four RNA extraction kits

<table>
<thead>
<tr>
<th>Onion Lots</th>
<th>Eluate negative control $C_t$</th>
<th>no matrix $C_t$</th>
<th>QIAamp Mini</th>
<th>QIAamp UltraSens</th>
<th>Mo Bio Ultraclean</th>
<th>MagMAX</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1.16</td>
<td></td>
</tr>
<tr>
<td>B</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>D</td>
<td>1.72</td>
<td>2.28</td>
<td>0.73</td>
<td>1.84</td>
<td></td>
<td></td>
</tr>
<tr>
<td>E</td>
<td>0</td>
<td>2.67</td>
<td>0.24</td>
<td>1.55</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*a The four extraction procedures of each lot were conducted separately with the same lot fresh green onions.

C. $C_t$ values corresponded to internal amplification control RNA detected were obtained per duplicate.

c Negative control samples were green onion eluates without MS2 inoculated.
4.2 Comparison of RNA Extraction Methods for the Detection of MS2 Coliphage from Concentrated Green Onions Eluates

4.2.1 Comparison of RNA Extraction Kits for Detection of MS2 and IAC RNA. Enteric viruses in food samples usually present at very low contamination levels. Moreover, the detection of viruses in food matrices can be hampered by the presence of inhibitory materials co-extracted with viral RNA. Therefore, it is necessary to concentrate and purify the low levels of viruses into a volume compatible with real-time RT-PCR analysis. In order to obtain better detection, the MS2 coliphage inoculated into centrifuged green onion eluates at 40 pfu/ml was concentrated using ultracentrifugation and then subjected to QIAshredder homogenization to remove and inactivate potential inhibitors prior to extraction by four commercially available RNA extraction methods.

The number of MS2 positives lots of five with and without QIAshredder pre-treatment is compared in Table 4.7. Detection of MS2 in green onion concentrated eluates without QIAshredder pre-treatment varied highly by kits with the Qiagen QIAamp Mini and QIAamp UltraSens kits allowing detection of 5/5 samples, whereas the Mo Bio UltraClean kit allowed detection of 4/5 and MagMAX only 1/5 samples. The addition of the QIAshredder homogenization treatment prior to RNA extraction resulted in an improvement for Mo Bio Ultraclean kit and MagMAX kit as the detection of MS2 positives increased to 5/5 samples, whereas the Qiagen QIAamp Mini and QIAamp UltraSens kits remained the same detection with 5/5 samples positive without QIAshredder pre-treatment. This data suggested that the use of the QIAshredder greatly reduced the inhibition of the PCR resulting from carryover of the matrix during extraction. The silica member spin-column-type extraction methods (QIAamp Mini, QIAamp UltraSens, and Mo Bio Ultraclean) generally performed better than the charged
magnetic bead-based extraction kit (MagMAX), though the use of the QIAshredder prior to extraction allowed detection of all samples across all kits.

The comparison of four viral RNA extraction methods for the detection of MS2 from green onion concentrated eluates with and without QIAshredder homogenization pre-treatment is shown in Figures 4.1 to 4.5 for each lot of green onions used for testing. For the Qiagen QIAamp Mini and QIAamp UltraSens kits, the detection of MS2 without QIAshredder pre-treatment was nearly equivalent to or had a lower Ct value than with QIAshredder pre-treatment. This was mirrored in the inhibition assay using the IAC. In contrast, for the detection of MS2 without QIAshredder pre-treatment, the Mo Bio Ultraclean kit had a nearly equal or higher Ct value compared to using QIAshredder pre-treatment. This was also mirrored in the inhibition assay using the IAC. And without prior treated by QIAshredder, this kit even failed to detect MS2 in onion lot D. On the other hand, without QIAshredder pre-treatment, the MagMAX kit failed to detect MS2 in all onion lots except onion lot A, and the inhibition assay using the IAC showed a higher Ct value or even no detection in both onion lots C and E compared to using QIAshredder pre-treatment. However, the addition of the QIAshredder pre-treatment gained detection for both the Mo Bio Ultraclean and MagMAX kits, and consequently resulted in positive detection in the MS2 and IAC assays for all five onion lots extracted by all extraction methods. These results suggested that though helpful in eliminating the real-time RT-PCR inhibitors for the Mo Bio Ultraclean and MagMAX extraction kits, the use of the QIAshredder appeared not to be necessary for good results with the Qiagen QIAamp Mini and QIAamp UltraSens kits when extracting RNA from green onion concentrated eluates.
The plausible reason of better detection for Qiagen QIAamp Mini and QIAamp UltraSens kits without QIAshredder pre-treatment may be that the use of the QIAshredder to remove inhibitory substances from concentrated sample matrix may simultaneously lead to the loss of viral particles, so that as more manipulation procedures are performed the fewer viral particles remain (Morales-Rayas, Wolffs, & Griffiths, 2010). However, concentrating the viral particles by ultracentrifugation may result in large pellets that can be difficult to dissolve (Rutjes, Lodder-Verschoor, van der Poel, van Duijnhoven, & Husmani, 2006; Rzezutka, D'Agostino, & Cook, 2006). The non-specific MagMAX positively charged magnetic beads may attract or bind inhibitors and debris from highly concentrated sample matrix when binding the viral particles, thus blocking detection from the MagMAX kit extracted samples. Therefore, the use of the QIAshredder prior to extraction is helpful in removing the real-time RT-PCR inhibitors and debris for the MagMAX extraction kit.

4.2.2 Effect of Extracted RNA Dilution on Detection of MS2 from Onion Eluates.

With real-time RT-PCR, 10-fold dilutions of the MagMAX extracted RNA without QIAshredder pre-treatment were applied in the amplification to reduce possible real-time RT-PCR inhibitors in the amplification reaction. The results shown in Table 4.8 indicate that with 10-fold diluted RNA, the MagMAX kit showed better detection in onion lots B, C and D without prior treated by QIAshredder. The Ct values of 10-fold diluted RNA in the MS2 assay were nearly equivalent to those seen when RNA was extracted with additional QIAshredder pre-treatment. This suggested that the dilution of the MagMAX extracted RNA probably had the same effect in removing real-time RT-PCR inhibitors as the QIAshredder pre-treatment and allowed detection for this kit down to 40 pfu/ml.
Recently, in a comparison of RNA extraction kits for detection of hepatitis A virus in strawberry samples, Bianchi, Dal Vecchio, Vilarino, and Romalde (2011) pointed out that, with RT-qPCR and ten-fold diluted RNA, all the kits tested showed an increase of sensitivity between 1-log unit and 3-log units. The resin column-based kits from Qiagen Rneasy Plant Mini kit, Talent Total Quick RNA Cells and Tissues Version Mini kit, and BioRad Aurum™ Total RNA Extraction kit allowed detection down to 0.05 pfu/mg of strawberry homogenate.

Based on the evaluation of the four commercial RNA extraction kits on green onion concentrated eluates inoculated at 40 pfu/ml, the Qiagen QIAamp Mini and MagMAX kits were selected for further evaluation. Of the silica spin-column-based kits, the QIAamp Mini was less inhibited compared to the QIAamp Ultrasens kit, and allowed better detection without QIAshredder pre-treatment compared to the Mo Bio Ultraclean kit. The magnetic bead-based MagMAX kit allowed the most inhibition; however, the use of QIAshredder was seen to reduce or eliminate inhibition of the real-time RT-PCR from green onion concentrated eluates and consequently improved detection. Therefore further comparison of the two kits at lower MS2 inoculation levels of 20 and 5 pfu/g was investigated.
Figure 4.1. Comparison of real-time RT-PCR cycle thresholds ($C_t$) for detection of MS2 and IAC targets after Lot A concentrated eluates were extracted with and without QIAshredder pre-treatment. Ultracentrifuged eluates were inoculated with MS2 at 40 pfu/ml prior to concentration and extraction. *=1/2° amplifications

Figure 4.2. Comparison of real-time RT-PCR cycle thresholds ($C_t$) for detection of MS2 and IAC targets after Lot B concentrated eluates were extracted with and without QIAshredder pre-treatment. Ultracentrifuged eluates were inoculated with MS2 at 40 pfu/ml prior to concentration and extraction. *=1/2° amplifications
Figure 4.3. Comparison of real-time RT-PCR cycle thresholds ($C_t$) for detection of MS2 and IAC targets after Lot C concentrated eluates were extracted with and without QIAshredder pre-treatment. Ultracentrifuged eluates were inoculated with MS2 at 40 pfu/ml prior to concentration and extraction. *=1/2* amplifications

Figure 4.4. Comparison of real-time RT-PCR cycle thresholds ($C_t$) for detection of MS2 and IAC targets after Lot D concentrated eluates were extracted with and without QIAshredder pre-treatment. Ultracentrifuged eluates were inoculated with MS2 at 40 pfu/ml prior to concentration and extraction. *=1/2* amplifications
Figure 4.5. Comparison of real-time RT-PCR cycle thresholds ($C_t$) for detection of MS2 and IAC targets after Lot E concentrated eluates were extracted with and without QIAshredder pre-treatment. Ultracentrifuged eluates were inoculated with MS2 at 40 pfu/ml prior to concentration and extraction. *=1/2* amplifications.
Table 4.7. Real-time RT-PCR detection of MS2 at 40 pfu/ml after eluate extraction by four RNA extraction kits with and without QIAshredder pre-treatment

<table>
<thead>
<tr>
<th>Methods</th>
<th>No.of MS2 positives/No.of total samples</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>QIAamp Mini</td>
</tr>
<tr>
<td>No QIAshredder</td>
<td>5/5</td>
</tr>
<tr>
<td>QIAshredder</td>
<td>5/5</td>
</tr>
</tbody>
</table>

Table 4.8. Ten-fold dilution effect on reduction of inhibition of real-time RT-PCR for MagMAX kit with and without QIAshredder pre-treatment

<table>
<thead>
<tr>
<th>Onion Lots</th>
<th>C&lt;sub&gt;t&lt;/sub&gt; values of MagMAX&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Undiluted RNA No QIAshredder</td>
</tr>
<tr>
<td>A</td>
<td>37.74±0.11</td>
</tr>
<tr>
<td>B</td>
<td>NA</td>
</tr>
<tr>
<td>C</td>
<td>NA</td>
</tr>
<tr>
<td>D</td>
<td>NA</td>
</tr>
<tr>
<td>E</td>
<td>ND&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup> C<sub>t</sub> values shown with standard deviation

<sup>b</sup> Sample was too viscous to pipet

ND=Not done
NA=No amplification
4.3 **Comparison of Qiagen QIAamp Mini and MagMAX Viral RNA Extraction Methods for the Detection of MS2 Coliphage from Green Onions**

Green onions were directly inoculated with MS2 coliphage at levels of 20 and 5 pfu/g, concentrated as before, and extracted by the Qiagen QIAamp Mini and MagMAX kits with and without prior treated by QIAshredder.

The number of MS2 positives from two onion lots processed with and without QIAshredder pre-treatment is compared in Table 4.9. Detection of MS2 on green onions varied highly by kits with the Qiagen QIAamp Mini allowing detection of 2/2 samples at both inoculation levels of 20 and 5 pfu/g regardless of the QIAshredder pre-treatment, whereas the MagMAX kit failed to detect MS2 at 20 and 5 pfu/g without the QIAshredder pre-treatment. The addition of the QIAshredder homogenization treatment prior to RNA extraction led to an improvement for the MagMAX kit as the detection of MS2 positive increased to 1/2 samples at 20 pfu/g, however, this kit still failed to detect MS2 at 5 pfu/g when prior treated by QIAshredder. These results indicated that the use of the QIAshredder could remove the inhibitor substances resulting from carryover of the matrix during extraction. The Qiagen QIAamp Mini kit performed better with the positive detection at 5 pfu/g, whereas the charged magnetic bead-based MagMAX kit only allowed detection of the MS2 RNA at an initial inoculation level of 20 pfu/g if processed with the QIAshredder.

The comparison of the Qiagen QIAamp Mini and MagMAX RNA extraction kits for the detection of MS2 on green onions with and without QIAshredder pre-treatment is shown in Figure 4.6 and Figure 4.7 for each lot of onions used for testing. When MS2 RNA was extracted using the Qiagen QIAamp Mini kit, without prior processing by the QIAshredder, the detection of MS2 at levels of 20 and 5 pfu/g was nearly equivalent to or
had a higher Ct value, however, in replicate amplifications compared to using QIAshredder pre-treatment. This was mirrored in the inhibition assay using the IAC. These results suggested that the use of the QIAshredder appeared not to be necessary for good results with the QIAamp Mini kit when extracting low inoculation levels of MS2 RNA on green onions. In contrast, without QIAshredder pre-treatment, the MagMAX kit failed to detect MS2 at 20 and 5 pfu/g in all onion lots, whereas, this kit did gain detection with additional QIAshredder pre-treatment at 20 pfu/g, but still failed to detect MS2 at 5 pfu/g. The inhibition assay using the IAC without QIAshredder pre-treatment showed a higher Ct value in onion lot B or even no detection in onion lot A compared to prior processed with QIAshredder. These results suggested that though helpful in eliminating the real-time RT-PCR inhibitors for the MagMAX kit at low inoculation level of 20 pfu/g, the use of QIAshredder did not improve the extraction efficiency of MagMAX kit when the MS2 RNA level was down to 5 pfu/g.

In a study by Butot, Putallaz, and Sánchez (2007) involving development of a rapid and sensitive method for detecting HAV, NV, and rotavirus (RV) from berries and vegetables, the silica spin-column-based Qaigen QIAamp Viral RNA Mini kit and the magnetic silica based bioMérieux Nuclisens magnetic kit were compared for extraction of viral RNA from inoculated frozen raspberries. The results indicated that both kits were suitable for HAV and NV detection in food samples, however, the bioMérieux kit failed to detect rotaviruses in berry samples. For this reason the Qiagen QIAamp Mini kit was chosen and viruses were eluted and concentrated by ultrafiltration. Using real-time RT-PCR, the average detection limits were 1 TCID$_{50}$, 54 RT-PCR units, and 0.02 TCID$_{50}$ per 15 g of food for HAV, NV, and RV, respectively. Compared to our results using the
ultracentrifugation to concentrate the viral particles with extraction by Qiagen QIAamp Mini kit, using real-time RT-PCR, the detection limit could be as low as 5 pfu/g for MS2 on green onions.
Figure 4.6. Comparison of real-time RT-PCR cycle thresholds (Ct) for detection of MS2 and IAC targets after onion Lot A was extracted with and without QIAshredder pretreatment. Green onions were inoculated at 20 and 5 pfu/g prior to concentration and extraction. * = 1/2 + amplifications

Figure 4.7. Comparison of real-time RT-PCR cycle thresholds (Ct) for detection of MS2 and IAC targets after onion Lot B was extracted with and without QIAshredder pretreatment. Green onions were inoculated at 20 and 5 pfu/g prior to concentration and extraction. * = 1/2 + amplifications
Table 4.9. Limit of Qiagen QIAamp Mini and MagMAX viral RNA extraction kits for detection of MS2 at low inoculation levels (20 and 5 pfu/g)

<table>
<thead>
<tr>
<th>MS2 Inoc. Level (pfu/g)</th>
<th>Treatment</th>
<th>No.of MS2 positives/No.of total samples</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>QIAamp Mini</td>
</tr>
<tr>
<td>20</td>
<td>No QIAshredder</td>
<td>2/2</td>
</tr>
<tr>
<td></td>
<td>QIAshredder</td>
<td>2/2</td>
</tr>
<tr>
<td>5</td>
<td>No QIAshredder</td>
<td>2/2</td>
</tr>
<tr>
<td></td>
<td>QIAshredder</td>
<td>2/2</td>
</tr>
</tbody>
</table>
CHAPTER 5
CONCLUSION AND RECOMMENDATIONS

5.1 Conclusion

The overall purpose of this research was to compare the ability of four commercially available nucleic acid extraction kits to extract and purify MS2 coliphage RNA from (1) green onion eluates, and (2) ultracentrifuge-concentrated eluates, with and without Qiagen QIAshredder pre-treatment, using detection by real-time RT-PCR.

From comparison of commercial viral RNA extraction kits for detecting of MS2 coliphage RNA in green onion eluates, a conclusion is drawn that the four kits are capable of detecting MS2 in at least 4/5 samples at $10^3$ and $10^2$ pfu/ml. Detection becomes sporadic at $10^1$ and $10^0$ pfu/ml with 1/5 or 2/5 samples shown MS2 positive with a single amplification in most kits. The greatest inhibition indicated by the internal amplification control (IAC) assay results from using MagMAX (3/5 lots with avg 1.5 Ct shift), followed by UltraSens (2/5 lots with avg 2.5 Ct shift), Mo Bio (2/5 lots with avg 0.5 Ct shift), and QIAamp Mini (1/5 lots with avg 1.7 Ct shift).

From this study of commercial viral RNA extraction kits for detecting MS2 in concentrated green onion eluates initially inoculated at 40 pfu/ml, we conclude that the use of the QIAshredder prior to extraction allows detection from all samples across all kits while greatly reducing the inhibition of the real-time RT-PCR resulting from carryover of the matrix during extraction. The spin-column-type extraction kits (QIAampMini, QIAamp UltraSens, and Mo Bio Ultraclean) generally perform better than the charged magnetic bead-based extraction kit (MagMAX). Therefore, QIAshredder pre-treatment does not appear to be necessary for good results with the Qiagen QIAamp Mini
and QIAamp UltraSens kits though helpful in removing the real-time RT-PCR inhibitors for the Mo Bio and MagMAX extraction kits when extracting from concentrated samples.

From evaluation of Qiagen QIAamp Mini and MagMAX viral RNA extraction kits for detecting ultracentrifuge-concentrated MS2 from green onions inoculated at 20 and 5 pfu/g, it is concluded that the Qiagen QIAamp Mini kit performs better with detection down to 5 pfu/g regardless of the additional QIAshredder treatment prior to extraction. The use of QIAshredder prior to extraction assists in eliminating the real-time RT-PCR inhibitors with the MagMAX kit at 20 pfu/g, however, the 5 pfu/g samples appear to be below the detection limit for this kit.

Based upon our study, we have found that RNA extracted with silica membrane spin-column-based methods seems to contain fewer inhibitors than those extracted by magnetic bead-based methods, and the addition of QIAshredder homogenization prior to extraction reduces the inhibition of the real-time RT-PCR resulting from carryover of the green onion matrices for the MagMAX kit, but does not appear to be necessary for the spin-column-based kits: QIAamp Mini, QIAamp UltraSens, and Mo Bio Ultraclean. The use of Qiagen QIAamp Viral Mini RNA Extraction kit, with real-time RT-PCR, can not only be labor and time saving but also helpful to detect enteric virus from green onion eluates with or without concentration and has proven to be a reliable tool during sample preparation for isolating and purifying enteric viruses eluted from fresh products.

5.2 Recommendations

Our future study would focus on comparing different nucleic acid extraction kits to extract and purify enteric virus from various food products, such as shellfish, fruits, and ready-to-eat vegetables. Since these commercial food products are often associated
with foodborne outbreaks, it will be necessary to know a standard concentration and extraction method that could offer a rapid and reliable sample preparation procedure for enteric virus detection. Moreover, in this present study, the effect of using the manufacturer’s recommended volumes for MS2 inoculated green onion eluates extraction is evaluated, but it would be interesting to know if standardizing the input and elution volumes has an effect on the co-extraction of inhibitors for final real-time RT-PCR outcome. The relevant information would be very helpful to both the food industry and the developers of test kits.
BIBLIOGRAPHY


