Development of Novel Methods for the Concentration and Detection of Foodborne Hepatitis A virus

by

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ABSTRACT

DEVELOPMENT OF NOVEL METHODS FOR THE CONCENTRATION AND DETECTION OF FOODBORNE HEPATITIS A VIRUS

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University of Guelph, 2014

Advisor:
Professor Mansel W. Griffiths

The low amounts of viral particles in contaminated foods make it challenging to develop methods for their detection. Rapid and easy-to-use methods for separating and concentrating viruses from contaminated foods are needed to enhance the efficiency of virus detection. In the present study, iron oxide (Fe₃O₄) magnetic nanoparticles (MNPs) coated with -NH₂ or protamine (designated NMNPs and PMNPs, respectively) were used to concentrate hepatitis A virus (HAV) from different food matrices. Successful coating of -NH₂ or protamine was confirmed using Fourier transform infrared spectroscopy (FTIR), zeta potential test, and transmission electron microscopy (TEM). Glycine buffer (0.05 M glycine, 0.14 M NaCl, 0.2% (v/v) Tween 20, pH 9.0) was found to be efficient in eluting HAV from artificially contaminated green onion, strawberry, and mussel. When used for concentrating HAV from the food eluates or milk, PMNPs showed higher sensitivity and repeatability than NMNPs. The detection limit of HAV by real-time RT-PCR was 8.3 plaque-forming unit (PFU)/15 g, 83 PFU/50 g, 8.3 PFU/5 g, and 8.3 PFU/40 mL for green onion, strawberry, mussel, and milk, respectively. Polyethylene glycol (PEG) dialysis method demonstrated similar analytical sensitivity to the PMNP method in concentrating HAV from green onion, mussel, and milk.
A bioluminescent real-time reverse transcriptase loop-mediated isothermal amplification (RT-LAMP-BART) technology was employed in the current study to detect HAV concentrated from green onion, strawberry, mussel, and milk by PMNPs. The analytical sensitivity of cDNA-LAMP-BART assay was 8.3 PFU/15 g, 83 PFU/50 g, 8.3 PFU/5 g, and 8.3 PFU/40 mL for green onion, strawberry, mussel, and milk, respectively. These results were comparable with those from real-time RT-PCR.

Qβ replicase reaction assay was also investigated in the present study for HAV detection. It was found that the method was not applicable in detecting foodborne virus due to the strong background signal in non-template reactions, which gave false positive results.
ACKNOWLEDGEMENT

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TABLE OF CONTENTS

LIST OF TABLES ...................................................................................................................... xv

LIST OF FIGURES ..................................................................................................................... xvi

LIST OF ABBREVIATIONS .......................................................................................................... xix

Chapter 1: INTRODUCTION AND LITERATURE REVIEW ....................................................... 1

1.1. Introduction of foodborne diseases ....................................................................................... 1

1.2. Introduction of foodborne viruses ......................................................................................... 11

1.2.1. Characteristics of foodborne viruses .............................................................................. 11

1.2.2. Categories of foodborne viruses ..................................................................................... 14

1.2.3. Transmission routes of viruses in food .......................................................................... 14

1.2.4. Prevention and control of foodborne viral illnesses ......................................................... 17

1.3. HAV .................................................................................................................................. 23

1.3.1. Characteristics of HAV .................................................................................................. 23

1.3.2. HAV infection ................................................................................................................ 26

1.3.3. Foodborne outbreaks of HAV ......................................................................................... 26

1.3.4. Stability of HAV ............................................................................................................ 30

1.3.4.1. Survival of HAV on animate and inanimate surfaces .................................................... 30

1.3.4.2. Persistence of HAV during food storage ...................................................................... 31

1.3.4.3. Resistance of HAV to chemical disinfectants .............................................................. 32

1.3.4.4. Resistance of HAV to thermal processing .................................................................. 34

1.3.4.5. Persistence of HAV against depuration process in shellfish ...................................... 36

1.3.4.6. Stability of HAV under non-thermal food processing conditions ............................... 38

1.4. Separation and concentration methods of foodborne viruses ............................................ 40
1.4.1. Methods of foodborne virus separation .......................................................... 41
1.4.2. Methods of foodborne virus concentration ...................................................... 42
  1.4.2.1. PEG precipitation ......................................................................................... 42
  1.4.2.2. Ultracentrifugation ..................................................................................... 45
  1.4.2.3. Ultrafiltration .............................................................................................. 47
  1.4.2.4. Charged membrane filtration ....................................................................... 49
  1.4.2.5. Magnetic bead separation ........................................................................... 53
1.4.3. Magnetic nanoparticle capture method ............................................................. 59
1.4.4. PEG dialysis method ....................................................................................... 65
1.5. Methods of foodborne virus detection ................................................................. 65
  1.5.1. Cell culture ....................................................................................................... 66
  1.5.2. Immunoassay .................................................................................................. 66
  1.5.3. Molecular biological methods ......................................................................... 67
    1.5.3.1. PCR technique .......................................................................................... 67
    1.5.3.2. Loop-mediated isothermal amplification (LAMP) technology .................. 70
    1.5.3.3. Qβ replicase reaction assay ....................................................................... 73
1.6. Objectives of this thesis ....................................................................................... 80

Chapter 2: FABRICATION OF CATIONIC MAGNETIC NANOPARTICLES FOR THE
CONCENTRATION OF HEPATITIS A VIRUS ................................................................. 82
  2.1. Introduction .......................................................................................................... 82
  2.2. Materials and methods ....................................................................................... 84
    2.2.1. Production of NH₂-coated Fe₃O₄ MNPs ........................................................... 84
    2.2.2. Production of COOH-coated Fe₃O₄ MNPs ..................................................... 86
2.2.3. Production of protamine-coated Fe$_3$O$_4$ MNPs.................................................................86

2.2.4. Characterization of chemically modified Fe$_3$O$_4$ MNPs..................................................87
  2.2.4.1. Fourier transform infrared spectroscopy (FTIR)...........................................................87
  2.2.4.2. Zeta potential analysis .....................................................................................................87
  2.2.4.3. Transmission electron microscopy (TEM).......................................................................87

2.2.5. HAV propagation ..................................................................................................................88

2.2.6. HAV plaque assay ................................................................................................................89

2.2.7. Real-time RT-PCR of HAV ................................................................................................90

2.2.8. Optimization of MNP capture conditions ...........................................................................91

2.2.9. Statistical analysis ..............................................................................................................93

2.3. Results and discussion ...........................................................................................................93
  2.3.1. Zeta potential analysis of chemically modified Fe$_3$O$_4$ MNPs .............................................93
  2.3.2. FTIR analysis of chemically modified Fe$_3$O$_4$ MNPs.........................................................96
  2.3.3. TEM analysis of protamine-coated MNPs .........................................................................99
  2.3.4. HAV propagation and quantification ...................................................................................99
  2.3.5. Recovery of HAV using MNPs under different pH, ionic concentrations, and MNP
         concentrations ......................................................................................................................100
    2.3.5.1. Optimization of pH .....................................................................................................100
    2.3.5.2. Optimization of NaCl concentration ..............................................................................102
    2.3.5.3. Optimization of MNP concentration .............................................................................103

2.4. Conclusions ...........................................................................................................................104

Chapter 3: A COMPARISON OF DIFFERENT METHODS FOR THE CONCENTRATION
OF HEPATITIS A VIRUS FROM DIFFERENT FOOD MATRICES ........................................105
3.1. Introduction .............................................................................................................. 105

3.2. Materials and methods .......................................................................................... 107
  3.2.1. Quantification of HAV RNA via real-time RT-PCR ........................................... 107
  3.2.2. Comparison of different RNA extraction methods .......................................... 108
  3.2.3. Separation and concentration of HAV from different foods artificially
         contaminated with HAV .................................................................................... 110
  3.2.4. Comparison of HAV recovery from inoculated and spiked sample using PMNPs
         ......................................................................................................................... 112
  3.2.5. Concentrating HAV by PEG dialysis method .................................................. 113
  3.2.6. Statistical analysis ........................................................................................... 114

3.3. Results ..................................................................................................................... 114
  3.3.1. Quantification of HAV via real-time RT-PCR .................................................. 114
  3.3.2. Comparison of different RNA extraction methods .......................................... 115
  3.3.3. Comparison of different methods for concentrating HAV from artificially
         contaminated green onions, strawberries, and mussels .................................... 116
  3.3.4. Comparison of HAV recovery from inoculated and spiked samples using
         protamine-coated MNPs ....................................................................................... 119
  3.3.5. Comparison of different methods for concentrating HAV from artificially
         contaminated milk ............................................................................................... 121

3.4. Discussion ............................................................................................................... 123

3.5. Conclusions ............................................................................................................. 131
Chapter 4: DETECTION OF FOODBORNE HEPATITIS A VIRUS USING A
BIOLUMISCENT REAL-TIME REVERSE TRANSCRIPTASE LOOP-MEDIATED
ISOTHERMAL AMPLIFICATION TECHNOLOGY .................................................. 132

4.1. Introduction ........................................................................................................ 132

4.2. Materials and methods ...................................................................................... 135

4.2.1. HAV propagation and RNA extraction .......................................................... 135

4.2.2. RT-LAMP of HAV ....................................................................................... 135

4.2.3. One-step RT-LAMP-BART assay ............................................................... 136

4.2.4. Two-step RT-LAMP-BART assay ............................................................... 138

4.2.5. Real-time RT-PCR analysis ......................................................................... 141

4.2.6. Initial processing of each food artificially contaminated with HAV ............. 141

4.2.7. Concentration of HAV using PMNPs ......................................................... 143

4.3. Results .................................................................................................................. 144

4.3.1. Real-time PCR of HAV ............................................................................... 144

4.3.2. RT-LAMP assay of HAV ............................................................................ 145

4.3.3. Optimization of Mg$^{2+}$ in RT-LAMP-BART assay of HAV ...................... 146

4.3.4. Comparison of HPLC-purified and cartridge-purified primers in RNA-LAMP-

BART assay ............................................................................................................ 147

4.3.5. Sensitivity analysis of RNA-LAMP-BART and cDNA-LAMP-BART assay..... 149

4.3.6. Comparison of real-time RT-PCR, RNA-LAMP-BART, and cDNA-LAMP-BART

assay for the detection of HAV from different foods .............................................. 151

4.4. Discussion .......................................................................................................... 152

4.5. Conclusions ....................................................................................................... 159
Chapter 5: APPLICATION OF Q-BETA REPLICASE REACTION ASSAY FOR THE DETECTION OF HEPATITIS A VIRUS ................................................................. 160

5.1. Introduction .................................................................................. 160

5.2. Materials and methods ................................................................. 161

5.2.1. Materials ................................................................................ 161

5.2.2. Construction of plasmids for synthesizing HAV-reporter probes ........................................ 163

5.2.2.1. Ligation of inserts with plasmid pUC-MDV-LR ........................................... 163

5.2.2.2. PCR analysis ........................................................................ 165

5.2.2.3. Plasmid transformation ........................................................ 165

5.2.3. RNA transcription .................................................................... 167

5.2.4. RNA replication by Qβ replicase ............................................. 168

5.2.5. Qβ replicase reaction assay protocol ......................................... 169

5.3. Results ......................................................................................... 172

5.3.1. Construction of recombinant plasmids ............................... 172

5.3.2. RNA transcription ..................................................................... 174

5.3.3. RNA replication by Qβ replicase ............................................. 178

5.4. Discussion .................................................................................. 180

5.5. Conclusions .............................................................................. 188

Chapter 6: GENERAL CONCLUSIONS AND FUTURE RESEARCH ....................... 189

6.1. General conclusions ................................................................. 189

6.2. Future research ......................................................................... 192

6.2.1. Concentration of different contaminants from food using charged MNPs ........ 193

6.2.2. Application of surface modified Fe₃O₄ MNPs for capturing pathogens in food... 194
6.2.3. Development of an easy-to-use apparatus to enhance the speed of PEG dialysis. 195

6.2.4. Further optimization of RT-LAMP-BART to improve the efficiency ............... 195

6.2.5. Evaluation of the effect of exogenous RNA on the sensitivity of RT-LAMP-BART
.................................................................................................................................................. 196

References .................................................................................................................................................. 197
LIST OF TABLES

Table 1.1. The major agents attributable to foodborne diseases. ..................................................2
Table 1.2. Cost of foodborne illness in the U.S. .................................................................9
Table 1.3. Characteristics of foodborne viruses. ............................................................13
Table 1.4. Selected foodborne HAV outbreaks. .................................................................28
Table 2.1. Effect of pH on HAV recovery rate using two cationic MNPs. .........................101
Table 2.2. Effect of NaCl concentration on HAV recovery rate using two cationic MNPs. ....102
Table 2.3. Effect of the concentration of protamine-coated MNPs on HAV recovery rate. ...103
Table 3.1. Comparison of different RNA extraction methods. ...........................................116
Table 3.2. Concentration of HAV from green onions, strawberries, and mussels using different methods ........................................................................................................119
Table 3.3. Recovery rate of HAV from inoculated and spiked samples using PMNPs. .......121
Table 3.4. Detection of HAV from milk pre-treated with different methods .......................122
Table 4.1. Details of primers used for HAV assays by real-time RT-PCR and RT-LAMP-BART ............................................................140
Table 4.2. Comparison of real-time RT-PCR, RNA-LAMP-BART, and cDNA-LAMP-BART assays in detecting HAV in green onions, strawberries, mussels, and milk .................................152
Table 5.1. BglIII digestion system for pUC-MDV-LR and insertion sequences ..................164
LIST OF FIGURES

Figure 1.1. The most and least risky water application methods for contaminated crops growing above ground with a little clearance.................................................................19

Figure 1.2. Schematic representation of HAV genome..................................................25

Figure 1.3. Schematic representation of the LAMP mechanism  (A) primer design for LAMP reaction; (B) starting structure producing steps of LAMP; (C) cycle amplification step..72

Figure 1.4. Genetic map of the phage Qβ.................................................................74

Figure 1.5. Secondary structure of MDV-1.................................................................78

Figure 2.1. Zeta potentials of NH₂-coated MNPs produced under different conditions. Each value is the mean of five replicates. Bars show standard deviation. .........................94

Figure 2.2. Zeta potentials of COOH-coated MNPs produced under different conditions. Each value is the mean of five replicates. Bars show standard deviation. .........................95

Figure 2.3. Zeta potentials of Fe₃O₄ MNPs with different coatings. Each value is the mean of five replicates. Bars show standard deviation. ........................................96

Figure 2.4. FTIR spectra of Fe₃O₄ MNPs with different coatings. .................................98

Figure 2.5. TEM images of uncoated (A) and protamine-coated (B) Fe₃O₄ MNPs. ........99

Figure 2.6. Standard curve of real-time RT-PCR made by 10-fold serial dilutions of HAV cDNA using ABI 7900HT system. Each value is the mean of three independent replicates. Bars show standard deviation.................................................................100

Figure 3.1. Standard curve of real-time RT-PCR made by 10-fold serial dilutions of HAV cDNA using ViiA 7 system. Each value is the mean of three independent replicates. Bars show standard deviation.................................................................115
Figure 4.1. Chemical mechanism of LAMP-BART assay. ................................................................. 134

Figure 4.2. Bison system (Lumora Ltd.) for LAMP-BART assays. ............................................ 139

Figure 4.3. Amplification of HAV cDNA in real-time RT-PCR monitored by the measurement of fluorescence................................................................. 144

Figure 4.4. Analysis of RT-LAMP amplicon of HAV RNA by 1% agarose gel electrophoresis. ................................................................. 145

Figure 4.5. Effect of MgSO₄ concentrations on RNA-LAMP-BART reaction of HAV........... 146

Figure 4.6. Effect of MgSO₄ concentrations on cDNA-LAMP-BART reaction of HAV....... 147

Figure 4.7. Comparison of HPLC-purified and cartridge-purified primers in RNA-LAMP-BART assay. ................................................................. 148

Figure 4.8. Standard curve of RNA-LAMP-BART assay generated by testing the time-to-peak of 10-fold serial dilutions of HAV RNA. Each value is the mean of three independent replicates. Bars show standard deviation. The assay was performed at 55°C for 100 min. ................................................................................................................................. 149

Figure 4.9. Standard curve of cDNA-LAMP-BART assay made by testing the time-to-peak of 10-fold serial dilutions of HAV cDNA. Each value is the mean of three independent replicates. Bars show standard deviation. The assay was performed at 62°C for 100 min. ................................................................................................................................. 150

Figure 5.1. The structure of plasmid pUC-MDV-LR................................................................. 162

Figure 5.2. Schematic chart showing the process of using the Qβ replicase reaction assay to detect virus genome................................................................. 171

Figure 5.3. Agarose gel electrophoretogram of PCR for recombinant plasmids. ............... 173
Figure 5.4. Agarose gel electrophoretogram of colony PCR for the plasmid pUC-MDV-HLP..
........................................................................................................................................173

Figure 5.5. Agarose gel electrophoretogram of colony PCR for the plasmid pUC-MDV-HSP..
........................................................................................................................................174

Figure 5.7. Agarose gel electrophoresis of transcribed RNAs. ..................................................176

Figure 5.8. The secondary structure of MDV-poly.. .................................................................176

Figure 5.9. The secondary structure of MDV-HSP.. .................................................................177

Figure 5.10. The secondary structure of MDV-HLP..................................................................177

Figure 5.11. RNAs amplified by Qβ replicase.. ........................................................................179

Figure 5.12. Agarose gel electrophoresis of amplified RNAs by Qβ replicase.........................179

Figure 5.13. Agarose gel electrophoresis of amplified product in no template control by Qβ replicase. ......................................................................................................................................180

Figure 6.1. FTIR spectra of Fe₃O₄ MNPs uncoated and coated with PGM. ............................194
# LIST OF ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>AMP</td>
<td>Adenosine monophosphate</td>
</tr>
<tr>
<td>AGMK</td>
<td>African green monkey kidney</td>
</tr>
<tr>
<td>APS</td>
<td>Adenosine 5’ phosphosulphate</td>
</tr>
<tr>
<td>APTES</td>
<td>3-Aminopropyltriethoxysilane</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>ATR</td>
<td>Attenuated total reflectance</td>
</tr>
<tr>
<td>BART</td>
<td>Bioluminescence assay in real-time</td>
</tr>
<tr>
<td>bp</td>
<td>Base pair</td>
</tr>
<tr>
<td>B-SC-1</td>
<td>Continuous cell line of AGMK</td>
</tr>
<tr>
<td>Bst</td>
<td><em>Geobacillus stearothermophilus</em></td>
</tr>
<tr>
<td>Cat</td>
<td>Catalogue</td>
</tr>
<tr>
<td>CCD</td>
<td>Charge-coupled device</td>
</tr>
<tr>
<td>CDC</td>
<td>Centers for Disease Control and Prevention of the United States</td>
</tr>
<tr>
<td>cDNA</td>
<td>Complementary DNA</td>
</tr>
<tr>
<td>CFU</td>
<td>Colony-forming unit</td>
</tr>
<tr>
<td>CMNP</td>
<td>COOH-coated magnetic nanoparticle</td>
</tr>
<tr>
<td>CPE</td>
<td>Cytopathic effect</td>
</tr>
<tr>
<td>Ct</td>
<td>Cycle threshold</td>
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<tr>
<td>Da</td>
<td>Dalton</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco's Modified Eagle Medium</td>
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<tr>
<td>DMF</td>
<td><em>N, N</em>-dimethylformamide</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>DTGS</td>
<td>Deuterated triglycine sulfate</td>
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<tr>
<td>E. coli</td>
<td><em>Escherichia coli</em></td>
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<tr>
<td>EDC</td>
<td>1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride</td>
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<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
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</tr>
<tr>
<td>EF</td>
<td>Elongation factor</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme-linked immunosorbent assay</td>
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<tr>
<td>FAM</td>
<td>6-carboxyfluorescein</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal bovine serum</td>
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<tr>
<td>FCV</td>
<td>Feline calicivirus</td>
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<tr>
<td>FRhK-4</td>
<td>Cloned line of fetal rhesus monkey kidney cells</td>
</tr>
<tr>
<td>FTIR</td>
<td>Fourier transform infrared spectroscopy</td>
</tr>
<tr>
<td>GAP</td>
<td>Good agricultural practices</td>
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<td>GMP</td>
<td>Good manufacturing practices</td>
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<td>HAV</td>
<td>Hepatitis A virus</td>
</tr>
<tr>
<td>HACCP</td>
<td>Hazard analysis and critical control point</td>
</tr>
<tr>
<td>HBV</td>
<td>Hepatitis B virus</td>
</tr>
<tr>
<td>HCV</td>
<td>Hepatitis C virus</td>
</tr>
<tr>
<td>HDA</td>
<td>Helicase-dependent isothermal amplification</td>
</tr>
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<tr>
<td>HSV-1</td>
<td>Herpes simplex 1 virus</td>
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<td>IEM</td>
<td>Immune electron microscopy</td>
</tr>
<tr>
<td>IR-FIFA</td>
<td>Infrared fluorescent immunofocus assay</td>
</tr>
<tr>
<td>Kb</td>
<td>Kilo bases</td>
</tr>
<tr>
<td>LAMP</td>
<td>Loop-mediated isothermal amplification</td>
</tr>
<tr>
<td>LB broth</td>
<td>Luria-Bertani broth</td>
</tr>
<tr>
<td>LED</td>
<td>Light-emitting diodes</td>
</tr>
<tr>
<td>LIFA</td>
<td>Luminescent immunofocus assay</td>
</tr>
<tr>
<td>MDV</td>
<td>Midvariant</td>
</tr>
<tr>
<td>MES</td>
<td>4-Morpholineethanesulfonic acid</td>
</tr>
<tr>
<td>MGB</td>
<td>Minor groove binder</td>
</tr>
</tbody>
</table>
MMBs  Magnetic microbeads
MNPs  Magnetic nanoparticles
MNV  Murine norovirus
MOI  Multiplicity of infection
MPN  Most probable number
NHS  N-hydroxysuccinimide
nt  Nucleotide
NMNP  NH$_2$-coated magnetic nanoparticle
NASBA  Nucleic acid sequence based amplification
NoV  Norovirus
NTP  Nucleotide triphosphate
ORF  Open reading frame
PBS  Phosphate buffered saline
PAA  Polyacrylamide
PCR  Polymerase chain reaction
PEI  Polyethyleneimine
PEG  Polyethylene glycol
PFU  Plaque-forming unit
pI  Isoelectric point
PLL  Poly-L-lysine
PMNP  Protamine-coated magnetic nanoparticle
PPI  Inorganic pyrophosphate
RIFA  Radioimmunofocus assay
RNA  Ribonucleic acid
RNase  Ribonuclease
rpm  Revolutions per minute
RT-PCR  Reverse transcription-polymerase chain reaction
s  Second
<table>
<thead>
<tr>
<th>Term</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Taq</td>
<td><em>Thermus aquaticus</em></td>
</tr>
<tr>
<td>TCID&lt;sub&gt;50&lt;/sub&gt;</td>
<td>50% Tissue culture infective dose</td>
</tr>
<tr>
<td>TEM</td>
<td>Transmission electron microscopy</td>
</tr>
<tr>
<td>µg</td>
<td>Microgram</td>
</tr>
<tr>
<td>µL</td>
<td>Microliter</td>
</tr>
<tr>
<td>UTR</td>
<td>Untranslated region</td>
</tr>
<tr>
<td>WHO</td>
<td>World Health Organization</td>
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</table>
Chapter 1: INTRODUCTION AND LITERATURE REVIEW

1.1. Introduction of foodborne diseases

Foodborne disease outbreaks are a growing public health concern worldwide. A foodborne disease outbreak is defined as the occurrence of two or more cases of a similar illness resulting from the ingestion of a common food (Gould et al., 2013). Foodborne diseases are the results of ingestion of food contaminated by a variety of bacteria, viruses, parasites, toxic chemicals, prions, as well as natural toxins such as those present in poisonous mushrooms (Dewaal et al., 2010; Hanson et al., 2012). The major agents attributable to foodborne diseases are listed in Table 1.1. Food contamination could occur at any stage in the process from food production to consumption. Common symptoms of foodborne diseases are mild and self-limiting diarrhea and vomiting, however, severe and life-threatening consequences such as kidney and liver failure, brain and neural disorders, miscarriage, cancer, septicemia and death are also present (Kuchenmüller et al., 2013). The World Health Organization (WHO) estimated that every year in the world, foodborne diarrheal diseases kill 2.2 million people, including 1.9 million children (Dewaal et al., 2010). Only a small portion of foodborne illnesses are confirmed by laboratory diagnosis and reported to public health authorities. Because sick individuals may not seek medical care, a specimen may not be submitted for laboratory diagnosis. The laboratory test does not identify a causative agent due to the limited diagnostic capacity, or the positive results are not reported to surveillance systems (Flint et al., 2005). This under-reporting is especially true when the foodborne illnesses are chronic, have mild symptoms, or occur in poor and remote areas where the food safety surveillance system is not available.
Table 1.1. The major agents attributable to foodborne diseases.

<table>
<thead>
<tr>
<th>Bacteria†</th>
<th>Viruses</th>
<th>Parasites</th>
<th>Chemicals</th>
<th>Toxins</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Bacillus cereus</em></td>
<td></td>
<td></td>
<td></td>
<td>α-chaconine</td>
</tr>
<tr>
<td><em>Brucella</em> spp.</td>
<td>Adenovirus</td>
<td><em>Cryptosporidium</em> spp.</td>
<td>Heavy metals</td>
<td>Aflatoxin B1</td>
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<tr>
<td><em>Campylobacter</em> spp.</td>
<td>Aichi virus</td>
<td><em>Giardia intestinalis</em></td>
<td>Organochlorine pesticides</td>
<td>Amanitins</td>
</tr>
<tr>
<td><em>Clostridium botulinum</em></td>
<td>Astrovirus</td>
<td><em>Toxoplasma gondii</em></td>
<td>Polychlorinated dibenzo-p-dioxins</td>
<td>Botulinum neurotoxins</td>
</tr>
<tr>
<td><em>Clostridium perfringens</em></td>
<td>Coronaviruses</td>
<td><em>Trichinella</em> spp.</td>
<td>Polybrominated diphenyl ethers</td>
<td>Cholera toxin</td>
</tr>
<tr>
<td>Diarrheagenic <em>Escherichia coli</em> other than STEC and ETEC</td>
<td>Enterovirus</td>
<td></td>
<td></td>
<td>Ciguatera</td>
</tr>
<tr>
<td>ETEC</td>
<td></td>
<td></td>
<td></td>
<td>Fumonisins</td>
</tr>
<tr>
<td><em>Listeria monocytogenes</em></td>
<td>Hepatitis A virus</td>
<td></td>
<td></td>
<td>Histamine</td>
</tr>
<tr>
<td><em>Mycobacterium bovis</em></td>
<td>Hepatitis E virus</td>
<td></td>
<td></td>
<td>Muscarine</td>
</tr>
<tr>
<td><em>Salmonella enterica</em> serotype Typhi</td>
<td>Norovirus</td>
<td></td>
<td></td>
<td>Ochratoxin A</td>
</tr>
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<td><em>Salmonella</em> spp., nontyphoidal</td>
<td>Rotavirus</td>
<td></td>
<td></td>
<td>Ricin</td>
</tr>
<tr>
<td><em>Shigella</em> spp.</td>
<td>Sapovirus</td>
<td></td>
<td></td>
<td>Staphylococcus-enterotoxins</td>
</tr>
<tr>
<td><em>Staphylococcus aureus</em></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>STEC non-O157</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>STEC O157</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Streptococcus</em> spp.</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Vibrio parahaemolyticus</em></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Vibrio vulnificus</em></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td><em>Vibrio cholerae</em></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Vibrio</em> spp., other</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Yersinia</em> enterocolitica</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

†ETEC, enterotoxigenic *Escherichia coli*; STEC, Shiga toxin-producing *Escherichia coli*. The content in the table is obtained from Arvanitoyannis et al. (2014), Chung et al. (2007), Friedman & Rasooly (2013), and Scallan et al. (2011b).
Most foodborne outbreaks are localized, for example, an outbreak after eating a meal at a restaurant. However, many outbreaks also occur in various places and some of them are even in global scale due to the widespread food distribution in modern society. In 2006, the WHO launched the initiative to estimate the global burden of foodborne diseases, and the burden of foodborne diseases worldwide was calculated according to age, sex, region, and causative agents by the end of 2013 (Kuchenmüller et al., 2013). However, the data were not publically accessible at the time this thesis was completed.

In the United States (U.S.), based on the U.S. population in 2006 (299 million persons), it was estimated that there were 48 million foodborne illnesses each year, responsible for 127,839 hospitalizations and 3,037 deaths (Scallan et al., 2011a). Among these illnesses, 9.4 million were caused by 31 known pathogens, resulting in 55,961 hospitalizations and 1,351 deaths, and 38.4 million were caused by unspecified agents, resulting in 71,878 hospitalizations and 1,686 deaths. It is estimated that 5.5 million (59%) foodborne illnesses were caused by noroviruses (NoVs), 3.6 million (39%) by bacteria, and 0.2 million (2%) by parasites. The most prevalent bacterial pathogens were nontyphoidal Salmonella spp. (1.0 million, 11%), Clostridium perfringens (1.0 million, 11%), and Campylobacter spp. (0.8 million, 9%) (Scallan et al., 2011b). By analyzing the data reported to U.S. Centers for Disease Control and Prevention (CDC) from 1998 to 2008, Gould et al. (2013) found that 13,405 foodborne disease outbreaks reported during that period resulted in 273,120 illnesses, 9,109 hospitalizations, and 200 deaths. Most outbreaks were caused by 8 pathogens including NoV (39%), Salmonella (26%), Shiga toxin–producing Escherichia coli (STEC) (6%), scombroid toxin/histamine (5%), Clostridium perfringens (5%), Staphylococcus enterotoxin (3%), ciguatoxin (3%), and
**Campylobacter jejuni** (2%). Most illnesses were associated with food prepared in restaurants or delis (68%), private homes (9%), and catering or banquet facilities (7%). The food commodities implicated mostly in the reported illnesses were poultry (18.9%), fish (18.6%), and beef (11.9%) (Gould et al., 2013). The pathogen-commodity pairs most commonly responsible for the illnesses were NoV and leafy vegetables (4,011 illnesses), *C. perfringens* and poultry (3,452 illnesses), *Salmonella* and vine-stalk vegetables (3,216 illnesses), and *C. perfringens* and beef (2,963 illnesses). The pathogen-commodity pairs responsible for the most hospitalizations were *Salmonella* and fruits/nuts (452 hospitalizations), *Salmonella* and vine-stalk vegetables (441 hospitalizations), STEC and beef (340 hospitalizations), and STEC and leafy vegetables (301 hospitalizations). The pathogen-commodity pairs responsible for the most deaths were *Listeria* and poultry (16 deaths), *Salmonella* and fruits/nuts (14 deaths), and STEC and leafy vegetables (7 deaths) (Gould et al., 2013).

In Canada, from 2005 to 2010, the annual estimates of foodborne illnesses were 4 million episodes, 1.6 million of which were attributed to 30 known pathogens and 2.4 million were related to unspecified agents (Thomas et al., 2013). The pathogens that caused the greatest number of illnesses were NoV (1 million illnesses), *C. perfringens* (177,000 illnesses), *Campylobacter* spp. (145,000 illnesses) and nontyphoidal *Salmonella* spp. (88,000 illnesses). These estimates were based on the Canadian population in 2006 (32.5 million persons) (Thomas et al., 2013).

In Australia, a foodborne disease surveillance system, OzFoodNet, was established by the Australian government in 2000 to aggregate and analyze the information about the incidence
of foodborne diseases annually at a national level (Ashbolt et al., 2001). In cooperation with the National Centre for Epidemiology and Population Health Australia, the OzFoodNet estimated that there were 5.4 million cases of foodborne illnesses annually in Australia circa 2000, resulting in approximately 15,000 hospitalizations and 80 deaths every year (Hall et al., 2005). NoV, *E. coli*, *Campylobacter* spp., and *Salmonella* spp. were estimated to cause the most illnesses. The estimates of foodborne diseases circa 2010 are underway currently (Green & Fitzsimmons, 2013). According to the annual report of the OzFoodNet network from 2001 to 2010, *S. Typhimurium* was the most common etiological agent identified in the foodborne outbreaks, restaurants were the most frequently reported food preparation setting, and the foods mostly linked to the outbreaks were poultry and eggs (Ashbolt et al., 2001; The OzFoodNet Working Group, 2002, 2003, 2004, 2005, 2006, 2007, 2008, 2009, 2010).

In New Zealand, from 2000 to 2009, over half a million estimates of foodborne illnesses were from the 24 specified pathogens annually based on analysis of the notifiable disease surveillance data from the EpiSurve database and referring the population (4.32 million persons) in 2009 (Creesey & Lake, 2011). For these illnesses, 59%, 39%, and 2% were due to bacteria, viruses, and parasites, respectively, with the major pathogens being NoV (39%), *Campylobacter* (34%), *C. perfringens* (12%), *Yersinia enterocolitica* (5%) and non-typhoidal *Salmonella* (4%). These foodborne illnesses resulted in 4,279 hospitalizations with NoV, *Campylobacter*, and non-typhoidal *Salmonella* accounting for 69%, 21%, and 4%, respectively (Creesey & Lake, 2011). These illnesses caused 17 deaths each year resulting from *L. monocytogenes* (35%), NoV (18%), and *Toxoplasma gondii* (18%). The unspecified
agents caused twice as many cases of foodborne illnesses as the 24 specified agents and more than four times as many hospitalizations as the specified agents (Creesey & Lake, 2011).

In the Netherlands, the surveillance data were available for 14 pathogens including Toxoplasma gondii, thermophilic Campylobacter spp., Shiga-toxin producing E. coli O157, non-typhoidal Salmonella spp., Cryptosporidium spp., Giardia spp., Bacillus cereus, C. perfringens, Staphylococcus aureus, L. monocytogenes, NoV, rotavirus, hepatitis A virus (HAV), and hepatitis E virus (HEV) (Havelaar et al., 2012). These pathogens caused 1.8 million cases of disease in 2009, 680,000 cases of which were attributable to foodborne transmission. The foodborne diseases resulted in 78 deaths that year. Among these pathogens, Toxoplasma gondii, thermophilic Campylobacter spp., NoV, rotavirus, and Salmonella spp. caused the highest burden of foodborne disease (Havelaar et al., 2012).

In Greece, from 1996 to 2006, 369,305 cases of foodborne illnesses per million inhabitants were estimated each year, resulting in 900 hospitalizations and 3 deaths per million inhabitants annually. Sixty-six percent of foodborne illnesses were caused by Brucella spp., Echinococcus, Salmonella, HAV, Clostridium botulinum, and Toxoplasma gondii. Forty-eight percent of deaths were caused by Brucella spp., and others by Salmonella, L. monocytogenes, Echinococcus, and Toxoplasma gondii (Gkogka et al., 2011).

In the United Kingdom, the foodborne diseases caused by Campylobacter kept increasing from 2000 to 2010, with 55,000 laboratory-confirmed cases of foodborne illnesses attributable to Campylobacter reported in 2008 and 60% – 80% of the cases linked to chicken (Food
Standards Agency 2011). Other pathogens most commonly involved in foodborne outbreaks included *L. monocytogenes*, *E. coli* O157, *Salmonella*, and NoV, with *E. coli* O157 outbreaks increasing slightly from 800 cases in 2000 to 1,100 cases in 2010, *L. monocytogenes* outbreaks increasing slightly from 150 cases in 2000 to 240 cases in 2010, NoV outbreak increasing dramatically from 5,000 cases in 2004 to 11,000 cases in 2010, and *Salmonella* outbreak decreasing consistently from 11,000 cases in 2000 to 6000 cases in 2010 (Food Standards Agency 2011). The number of reported laboratory-confirmed cases of foodborne illness was known to be lower than the actual number of outbreaks due to under-reporting of cases. The under-reporting rate may vary between only one in three to one in a hundred. To account for this, the Health Protection Agency conducted the estimates of foodborne disease in England and Wales in 2008 and results showed that the estimates of foodborne illness caused by *Campylobacter*, NoV, *C. perfringens*, *Salmonella*, *E. coli* O157, and *L. monocytogenes* were 321,179; 201,279; 52,530; 26,962; 1,054; and 358, respectively. The highest number of deaths was caused by *L. monocytogenes* (126 deaths), followed by *Salmonella* (77 deaths), *Campylobacter* (76 deaths), *C. perfringens* (55 deaths), NoV (32 deaths), and *E. coli* O157 (23 deaths) (Food Standards Agency 2011).

By aggregating the data published in 2,314 papers, Xue & Zhang (2013) reported that from 1999 to 2010, there were 2,387 foodborne outbreaks in China, resulting in 99,487 foodborne illnesses and 380 deaths. Among these foodborne illnesses, 66,247 (66.6%) were caused by bacteria, 14,921 (15%) by chemicals, 7,065 (9.2%) by food toxins, and 7,648 (7.7%) by unspecified agents. The most deaths (53.4%) were caused by chemicals. The number of deaths caused by bacteria and fungi were 19.7% and 16.8%, respectively. The 10 most prevalent
bacterial pathogens, *Vibrio parahemolyticus*, *Salmonella*, *Proteus vulgaris*, *B. cereus*, *Staphylococcus*, *E. coli*, *Shigella*, *C. botulinum*, *Vibrio alginolyticus* and *Aeromonas*, were found linked to 64.6% of the illnesses and 16% of the deaths. *C. botulinum* was found to be the deadliest bacterium, responsible for 11.6% of the deaths. Animal-based food, such as seafood, poultry, pork, and beef, was the leading food vehicles, accounting for 52.2% of the illnesses. The highest numbers of illnesses were linked with school cafeterias (26.7%), restaurants (23.3%), rural family banquets (13%), company cafeterias (12.6%), and private homes (11.3%) (Xue & Zhang, 2013).

The foodborne diseases cause considerable economic loss to the society. The costs of foodborne diseases include costs of regulation and surveillance incurred by the government, costs associated with loss of productivity due to worker absenteeism, costs of health care, costs of food recall and disposal, costs of income loss due to closure of businesses, and costs of sale loss when consumers avoid particular products (Hussain & Dawson, 2013). The cost of foodborne illnesses in the U.S. is shown in Table 1.2. It is estimated that an annual total of $152 billion is spent on foodborne illnesses in the U.S. (Scharff 2010). The burden of foodborne diseases in Australia costs approximately $1.2 billion dollars annually (Hall et al., 2005). In New Zealand, the estimated total costs of foodborne diseases were $161.9 million in 2009 (Gadiel 2010). In England and Wales, foodborne disease has cost about £1.5 billion per year from 2005 to 2008 (Gormley et al., 2011). In developing countries, the problem of foodborne disease is far greater and the effect of the disease on economic activity can be far more severe.
Table 1.2. Cost of foodborne illness in the U.S. (Scharff 2010).

<table>
<thead>
<tr>
<th>Pathogens</th>
<th>Hospital services ($)</th>
<th>Physician services ($)</th>
<th>Drugs ($)</th>
<th>Deaths ($)</th>
<th>Quality of life ($)</th>
<th>Total cost per case ($)</th>
<th>Cases</th>
<th>Total cost to U.S. residents ($ million)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Bacterial</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bacillus cereus</td>
<td>4</td>
<td>21</td>
<td>3</td>
<td>0</td>
<td>198</td>
<td>226</td>
<td>29,439</td>
<td>7</td>
</tr>
<tr>
<td>Botulism</td>
<td>157,703</td>
<td>1885</td>
<td>37</td>
<td>542,012</td>
<td>24,726</td>
<td>726,362</td>
<td>62</td>
<td>45</td>
</tr>
<tr>
<td>Brucella spp.</td>
<td>3,692</td>
<td>107</td>
<td>5</td>
<td>60,689</td>
<td>6,206</td>
<td>70,698</td>
<td>818</td>
<td>58</td>
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<tr>
<td>Campylobacter spp.</td>
<td>137</td>
<td>33</td>
<td>5</td>
<td>616</td>
<td>8,110</td>
<td>8,901</td>
<td>2,112,302</td>
<td>18,803</td>
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<tr>
<td>Clostridium perfringens</td>
<td>2</td>
<td>21</td>
<td>3</td>
<td>221</td>
<td>263</td>
<td>510</td>
<td>267,403</td>
<td>136</td>
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<tr>
<td>E. coli O157:H7</td>
<td>921</td>
<td>54</td>
<td>4</td>
<td>12,460</td>
<td>1,339</td>
<td>14,838</td>
<td>66,905</td>
<td>993</td>
</tr>
<tr>
<td>E. coli, Non-O157 STEC</td>
<td>6</td>
<td>21</td>
<td>3</td>
<td>0</td>
<td>1,309</td>
<td>1,339</td>
<td>5,368</td>
<td>7</td>
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<tr>
<td>E. coli, Other</td>
<td>5</td>
<td>21</td>
<td>3</td>
<td>0</td>
<td>1,339</td>
<td>1,368</td>
<td>4,422</td>
<td>6</td>
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<tr>
<td>Listeria monocytogenes</td>
<td>78,127</td>
<td>1541</td>
<td>43</td>
<td>1,573,209</td>
<td>42,222</td>
<td>1,695,143</td>
<td>5,205</td>
<td>8,823</td>
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<tr>
<td>Salmonella, Typhi</td>
<td>21,641</td>
<td>816</td>
<td>35</td>
<td>35,767</td>
<td>4,251</td>
<td>62,509</td>
<td>536</td>
<td>34</td>
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<td>Salmonella, nontyphoidal</td>
<td>278</td>
<td>35</td>
<td>5</td>
<td>3,239</td>
<td>5,590</td>
<td>9,146</td>
<td>1,597,411</td>
<td>14,609</td>
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<tr>
<td>Shigella spp.</td>
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<td>34</td>
<td>5</td>
<td>1,227</td>
<td>5,611</td>
<td>7,092</td>
<td>96,686</td>
<td>686</td>
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<tr>
<td>Staphylococcus</td>
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<td>25</td>
<td>3</td>
<td>85</td>
<td>601</td>
<td>818</td>
<td>199,121</td>
<td>163</td>
</tr>
<tr>
<td>Streptococcus</td>
<td>93</td>
<td>24</td>
<td>3</td>
<td>0</td>
<td>2,167</td>
<td>2,288</td>
<td>54,789</td>
<td>125</td>
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<tr>
<td>Vibrio cholera, toxigenic</td>
<td>3485</td>
<td>228</td>
<td>16</td>
<td>0</td>
<td>1,669</td>
<td>5,428</td>
<td>52</td>
<td>&lt; 1</td>
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<tr>
<td>Vibrio vulnificus</td>
<td>34,950</td>
<td>595</td>
<td>42</td>
<td>3,009,896</td>
<td>243</td>
<td>3,045,726</td>
<td>51</td>
<td>154</td>
</tr>
<tr>
<td>Vibrio, other</td>
<td>152</td>
<td>27</td>
<td>3</td>
<td>19,947</td>
<td>1,681</td>
<td>21,810</td>
<td>5,511</td>
<td>120</td>
</tr>
<tr>
<td>Yersinia enterocolitis</td>
<td>293</td>
<td>35</td>
<td>5</td>
<td>181</td>
<td>6,713</td>
<td>7,227</td>
<td>93,321</td>
<td>674</td>
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<tr>
<td><strong>Parasitic</strong></td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cryptosporidium parvum</td>
<td>126</td>
<td>25</td>
<td>3</td>
<td>1,834</td>
<td>2,436</td>
<td>4,424</td>
<td>46,978</td>
<td>208</td>
</tr>
<tr>
<td>Cyclospora cayetanensis</td>
<td>19</td>
<td>21</td>
<td>3</td>
<td>0</td>
<td>1,489</td>
<td>1,531</td>
<td>32,322</td>
<td>49</td>
</tr>
<tr>
<td>Giardia lamblia</td>
<td>44</td>
<td>22</td>
<td>3</td>
<td>39</td>
<td>3,567</td>
<td>3,675</td>
<td>175,033</td>
<td>643</td>
</tr>
<tr>
<td>Toxoplasma gondii</td>
<td>1,280</td>
<td>49</td>
<td>3</td>
<td>26,197</td>
<td>1,889</td>
<td>29,429</td>
<td>121,048</td>
<td>3,562</td>
</tr>
<tr>
<td>Trichinella spiralis</td>
<td>3,224</td>
<td>87</td>
<td>5</td>
<td>0</td>
<td>8,548</td>
<td>11,864</td>
<td>56</td>
<td>1</td>
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</tbody>
</table>

(Continued on next page)
### Table 1.2. (continued)

<table>
<thead>
<tr>
<th>Viral Group</th>
<th>Cases</th>
<th>Males</th>
<th>Females</th>
<th>Cases</th>
<th>Males</th>
<th>Females</th>
<th>Total Cases</th>
<th>Total Deaths</th>
<th>Total Deaths Rate</th>
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<tr>
<td>Noroviruses</td>
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<td>22</td>
<td>3</td>
<td>106</td>
<td>413</td>
<td>586</td>
<td>9,899,026</td>
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<td>96</td>
<td>27</td>
<td>3</td>
<td>0</td>
<td>1,028</td>
<td>1,155</td>
<td>41,963</td>
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<td>1,202</td>
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<td>41,963</td>
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<td>Hepatitis A virus</td>
<td>495</td>
<td>36</td>
<td>3</td>
<td>7,540</td>
<td>3,119</td>
<td>11,193</td>
<td>41,963</td>
<td>10</td>
<td></td>
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<tr>
<td>Unknown agents</td>
<td>76</td>
<td>23</td>
<td>3</td>
<td>429</td>
<td>898</td>
<td>1,430</td>
<td>67,012,10</td>
<td>95,806</td>
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<tr>
<td>All illnesses</td>
<td>1851</td>
<td>81,910,77</td>
<td>151,626</td>
<td></td>
<td></td>
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</tr>
</tbody>
</table>
1.2. Introduction of foodborne viruses

1.2.1. Characteristics of foodborne viruses

Unlike bacteria that generally have sizes from 0.5 to 5 micrometers, viruses are very small microorganisms, ranging in size from 15 to 500 nanometers (Flint et al., 2004; Koopmans & Duizer, 2004). Virus genome can be DNA or RNA, in double-stranded (ds) or single-stranded (ss) form. The virus particle can vary from a simple structure consisting of a protein coat encompassing a genome to a rather complex structure consisting of an envelope membrane covering a complex protein capsid and a segmented genome (Flint et al., 2004). The structure of a virus particle is linked to the environmental resistance of the virus, with the more complex structure less resistant. Most foodborne viruses are with non-enveloped structure and they are quite stable outside of the host and resistant to harsh environmental conditions (Koopmans & Duizer, 2004). Viruses can survive on hard surfaces, such as door handles, light switches, and refrigerator door handles, for days or weeks, and are capable of contaminating things that touch those surfaces (D'Souza et al., 2006; Koopmans & Duizer, 2004). In comparison to bacteria, viruses do not contain all the biochemical mechanisms for their own replication and need to replicate inside specific living cells. For this reason viruses cannot replicate in foodstuffs and therefore cannot cause deterioration and organoleptic change of contaminated foods. Even though the number of viruses in contaminated foods is usually low due to the incapacity of viruses to reproduce themselves in foods, the low level of viruses in food can be very harmful for consumers since illnesses can be induced by a small number of viral particles (1 – 100) (Koopmans & Duizer, 2004). Teunis et al. (2008) reported that the infectious dose of human NoV was as few as 18 particles.
In the past decade, foodborne viral diseases have been increasingly recognized, with NoV and HAV mostly involved in foodborne outbreaks. In the U.S., NoVs are responsible for most of the epidemic acute gastroenteritis, causing 56,000 – 71,000 hospitalizations, 570 – 800 deaths, 400,000 emergency department visits, 1.7 – 1.9 million outpatient visits, and 19 – 21 million total illnesses each year (Hall et al., 2013). In Canada, the annual number of foodborne NoV outbreaks is from 300 to 400 (Public Health Agency of Canada, 2012). In the U.S., the annual outbreaks of HAV decreased from 13,397 in 2000 to 1,670 in 2010 (CDC, 2012). In Canada, between 1000 and 3000 cases of hepatitis are reported every year. Hepatitis A accounts for 25% – 40% of all cases of acute hepatitis with one in four cases requiring hospitalization and the mortality rate among hospitalized adult cases has been estimated to be 0.15% (Costa-Mattioli et al., 2003). However, the infections caused by HAV in food are still very common in the world. Recently, there have been several large outbreaks of HAV worldwide. In 2013, over 800 people were sick in Italy due to consumption of contaminated frozen berries (http://barfblog.com/tags/europe/). And 165 individuals from 10 states in the U.S. were infected by HAV from the consumption of contaminated pomegranate seeds (Collier et al., 2014). WHO estimates an annual 1.5 million clinical cases of hepatitis A worldwide (Wasley et al., 2006). In addition to NoV and HAV, other viruses (Table 1.3) frequently transmitted by food include HEV, human rotavirus, adenovirus, astrovirus, aichi virus, sapovirus, and enterovirus (Atreya, 2004; Koopmans & Duizer, 2004). The newly emerging viruses potentially transmitted through food include highly pathogenic avian influenza virus A (H5N1), severe acute respiratory syndrome coronavirus (SARS-CoV), and Nipah virus (Newell et al., 2010).
<table>
<thead>
<tr>
<th>Virus genus or species</th>
<th>Family</th>
<th>Genome type</th>
<th>Genome size (kb)</th>
<th>Capsid structure</th>
<th>Particle size (nm)</th>
<th>pI</th>
<th>Envelope</th>
<th>Culturable</th>
<th>Disease</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adenovirus</td>
<td>Adenoviridae</td>
<td>dsDNA</td>
<td>25 – 48</td>
<td>Icosahedral</td>
<td>70 – 90</td>
<td>4.5</td>
<td>N</td>
<td>Y†</td>
<td>Respiratory and gastroenteritis</td>
</tr>
<tr>
<td>Aichi virus</td>
<td>Picornaviridae</td>
<td>(+)ssRNA</td>
<td>8.3</td>
<td>Icosahedral</td>
<td>27 – 30</td>
<td>ND§</td>
<td>N</td>
<td>Y</td>
<td>Gastroenteritis</td>
</tr>
<tr>
<td>Astrovirus</td>
<td>Astroviridae</td>
<td>(+)ssRNA</td>
<td>7 – 8</td>
<td>Icosahedral</td>
<td>28 – 30</td>
<td>ND§</td>
<td>N</td>
<td>Y†</td>
<td>Gastroenteritis</td>
</tr>
<tr>
<td>Coxsackievirus</td>
<td>Picornaviridae</td>
<td>(+)ssRNA</td>
<td>7.4</td>
<td>Icosahedral</td>
<td>29</td>
<td>6.6 – 8.2</td>
<td>N</td>
<td>Y†</td>
<td>Meningitis</td>
</tr>
<tr>
<td>Echovirus</td>
<td>Picornaviridae</td>
<td>(+)ssRNA</td>
<td>7.5</td>
<td>Icosahedral</td>
<td>24 – 30</td>
<td>4.0 – 6.4</td>
<td>N</td>
<td>Y</td>
<td>Meningitis</td>
</tr>
<tr>
<td>H5N1</td>
<td>Orthomyxoviridae</td>
<td>(-)ssRNA</td>
<td>13.5</td>
<td>Helical</td>
<td>80 – 120</td>
<td>6.8</td>
<td>Y</td>
<td>Y</td>
<td>Influenza</td>
</tr>
<tr>
<td>Hepatitis A virus</td>
<td>Picornaviridae</td>
<td>(+)ssRNA</td>
<td>7.5</td>
<td>Icosahedral</td>
<td>27 – 32</td>
<td>2.8</td>
<td>N</td>
<td>Y†</td>
<td>Hepatitis</td>
</tr>
<tr>
<td>Hepatitis E virus</td>
<td>Hepeviridae</td>
<td>(+)ssRNA</td>
<td>7.2</td>
<td>Icosahedral</td>
<td>32 – 34</td>
<td>ND§</td>
<td>N</td>
<td>Y</td>
<td>Hepatitis</td>
</tr>
<tr>
<td>Nipah</td>
<td>Paramyxoviridae</td>
<td>(-)ssRNA</td>
<td>18.2</td>
<td>Helical</td>
<td>120 – 500</td>
<td>ND§</td>
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<td>Y</td>
<td>Encephalitis</td>
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<tr>
<td>Norovirus</td>
<td>Caliciviridae</td>
<td>(+)ssRNA</td>
<td>7.4 – 7.7</td>
<td>Icosahedral</td>
<td>28 – 35</td>
<td>5.5 – 6.9</td>
<td>N</td>
<td>N</td>
<td>Gastroenteritis</td>
</tr>
<tr>
<td>Poliovirus</td>
<td>Picornaviridae</td>
<td>(+)ssRNA</td>
<td>7.4</td>
<td>Icosahedral</td>
<td>29</td>
<td>4.5 – 7.2</td>
<td>N</td>
<td>Y†</td>
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<td>Reoviridae</td>
<td>dsRNA</td>
<td>16 – 27</td>
<td>Icosahedral</td>
<td>60 – 80</td>
<td>8.0</td>
<td>N</td>
<td>Y</td>
<td>Gastroenteritis</td>
</tr>
<tr>
<td>Sapovirus</td>
<td>Caliciviridae</td>
<td>(+)ssRNA</td>
<td>7.4 – 7.7</td>
<td>Icosahedral</td>
<td>28 – 35</td>
<td>5.0</td>
<td>N</td>
<td>N</td>
<td>Gastroenteritis</td>
</tr>
<tr>
<td>SARS</td>
<td>Coronaviridae</td>
<td>(+)ssRNA</td>
<td>29.7</td>
<td>Helical</td>
<td>80 – 140</td>
<td>ND§</td>
<td>Y</td>
<td>Y</td>
<td>Respiratory disease</td>
</tr>
</tbody>
</table>

†Not all strains within the genus are culturable and wild-type strains are often difficult to culture; † dsDNA means double-stranded DNA; dsRNA means double-stranded RNA; (+)ssRNA means positive-sense single-stranded RNA; (-)ssRNA means negative-sense single-stranded RNA; § ND means Not determined; The content in this table is a summary of reported data from Chang et al. (2006); Chang et al. (2007); Di Martino et al. (2013); Ferguson et al. (2003); Goodridge et al. (2004); Greening (2006); Guo et al. (2013); Luby et al. (2006); and Michen & Graule (2010).
1.2.2. Categories of foodborne viruses

Based on the symptoms of viral infection, foodborne viruses can be characterized into four different groups (Koopmans & Duizer, 2004). The first group includes viruses that infect human intestinal tract and cause gastroenteritis such as NoV, rotavirus, astrovirus, Aichi virus, adenovirus and sapovirus. The second group includes HAV and HEV. They are transmitted enterically and manifest disease in the liver. The third group includes viruses that replicate in the human intestine and only cause illness after migrating to the central nervous system, such as enterovirus including poliovirus, coxsackievirus, and echovirus. The last group includes the new emerging Nipa virus, HPAI-H5N1, and SARS-CoV. They infect human respiratory tract, are present in the intestinal tracts of infected humans and animals, and are shed into environment through feces. The viruses in this group cause pneumonia, respiratory failure, and death (Newell et al., 2010).

1.2.3. Transmission routes of viruses in food

Almost any kind of food can be involved in virus transmission. However, a limited number of foods are commonly associated with outbreaks. These include raw or under-cooked shellfish and meat, fresh produce and vegetables, and ready-to-eat foods such as sandwiches, salads, and sushi, etc. (Le Guyader & Atmar, 2008). These foods can be contaminated with viruses through the following three different major routes.

The first route is through human sewage-contaminated water. It was reported that methods normally used for sewage treatment could not effectively inactivate viruses and the treated sewage was still positive for enteric viruses (Okoh et al., 2010). Shellfish may be grown in
sewage-contaminated water and accumulate viral particles inside their bodies. Fresh produce can be tainted with viruses through the use of sewage-contaminated water in irrigation, washing, and preparation for pesticide or as fertilizer. Enteric viruses may be able to internalize into plant tissues through both the cut edge and stomata on the edible portions and through root uptake (Hirneisen et al., 2012; Wei et al., 2010). Stine et al. (2011) reported that 0.00021% – 9.4 % of *Salmonella* and 0.055% – 4.2% of HAV could be transferred from pesticides prepared using pathogen-contaminated water to the surface of fresh produce in spraying process. Virus transmission can occur when contaminated water is used to reconstitute food products such as dried or powered milk, infant formula, or juice. Rotavirus is a major concern for children’s health. The reconstitution of baby food with rotavirus-contaminated water might be the cause of virus transmission (Newell et al., 2010). The packing ice, if made from contaminated water, can also be a source of virus contamination of food.

The second route is through infected food handlers (Bidawid et al., 2004). The infected individuals shed viral particles in their feces before the appearance or after the vanishment of the symptoms. Typically, at least $10^6$ viral particles could be secreted in one gram of stool of the infected individuals (La Rosa et al., 2013). Up to $10^{11}$ of viral particles per gram stool were reported for rotavirus-infected persons (Koopman & Duizer, 2004). The hand of food handlers may become contaminated with viruses if the handlers do not practice appropriate personal hygiene when they clean toilet areas or change diapers. Therefore, the infected food handlers could transmit viruses to foods unknowingly at the time of food preparation. Virus contamination due to human handling can occur at any stage of the farm-to-fork continuum,
including before harvest, during processing, packing, distribution, and the time of consumption. The prepared ready-to-eat foods are usually contaminated with viruses in this mode. The infected food handlers could transmit viruses to shellfish meat when doing shucking. The infected pickers could transmit viruses to fresh produce in harvest.

The third route of virus transmission is zoonotic. One example of such virus is HEV that is classified in the family *Hepeviridae* and genus *Hepevirus*. The virus mainly includes four genotypes (I, II, III and IV) and all of them infect humans with the virus of genotype I also infecting swine, of genotype III also infecting swine, deer, mongoose, and rabbits, and of genotype IV also infecting swine, cattle, and sheep (Meng, 2011). It was reported that the virus sequences recovered from commercial pig livers were closely related to, or identical in a few cases, to the viruses recovered from human hepatitis E patients (Yazaki et al., 2003). In a case-control study in France, 7 of 13 individuals from three families who ate raw figatellu pig liver sausages became infected by HEV, while 5 other individuals from the same families who did not eat the figatellu sausages were not infected (Colson et al., 2010). Nipah virus is transmitted from fruit bats, through date palm sap, to human. Fruit bats frequently visit date palm trees and lick the sap during collection, potentially contaminating the sap with the virus from saliva or urine. The consumption of contaminated raw date palm sap or the alcoholic beverages made from the sap is the main risk factor for human infection (Khan et al., 2012). The disease caused by this virus is predominantly in Bangladesh. Up to January 2014, there have been 304 reported human cases linked to Nipah virus in Bangladesh with 232 deaths, giving a reported fatality rate of 76% (Simons et al., 2014). HPAI-H5N1 has been detected in undercooked poultry eggs and meat. Although human infections are mostly due to direct
contact with live infected birds, the discovery of the virus in poultry meat and eggs raises the concern that the virus has the potential to be transmitted to humans when undercooked meat and eggs are consumed (Chmielewski & Swayne, 2011). Between November 2002 and July 2003, SARS-CoV caused an epidemic outbreak in China, resulting in 8,098 infections and 774 deaths worldwide (Mckinney et al., 2006). The virus was spread into the human population through the preparation and potential consumption of food animals that appeared to have contracted the infection from another reservoir, probably bats (Lau et al., 2005).

1.2.4. Prevention and control of foodborne viral illnesses

Most viruses can survive in food exceeding the shelf life of the products. The survival time of virus is usually much longer than that of bacterial contaminants. In most countries, the microbiological quality control criteria for food rely on standard counts of coliform bacteria. These criteria are insufficient to protect against viral food-borne infections. For example, in the depuration process of shellfish, bacterial contaminants could be removed more efficiently than viruses (Schwab et al., 1998). It is, therefore, important to design strategies capable of controlling viral pathogens in food. Foodborne viruses are resistant to food processing conditions. Thus, for controlling foodborne viral illnesses, prevention of contamination should be the focus, rather than destruction of the contaminated viruses using various food-processing methods. According to the aforementioned transmission routes of viruses in food, the following strategies have been suggested for the prevention of foodborne viral illnesses.

Since human sewage-contaminated water is a major route of viral contamination in shellfish, prevention of shellfish growing areas from sewage contamination is of great importance. The
shellfish industry, wastewater treatment authorities, the public health authorities, and the food safety authorities need to collaborate with each other to protect the shellfish growing areas. Best Aquaculture Practices (BAP) should be followed to ensure the safety of seafood (Global Aquaculture Alliance, 2014). The aquaculture site should not be established in areas susceptible to sewage contamination. Illegal waste discharge from boats or sewage-treatment systems should be avoided. The routine sanitary surveillance of the shellfish growing area should be performed to monitor the water quality and identify possible pollution sources. The efforts should be made to control the pollution. The harvest areas should be closed as needed and warnings should be issued to the public when laboratory analysis indicates shellfish are unsafe to eat. Educating the public regarding appropriate disposal of human wastes, especially near the vicinity of shellfish-growing area, and the hazard of eating raw or undercooked shellfish is the most effective preventive measure.

Human sewage contaminated-water used for irrigation and fertilization in the pre-harvest stage or washing in the post-harvest stage is also a major concern for viral contamination of fresh produce. Water used for irrigation must be of high quality. Water with significant risks of virus contamination needs to be decontaminated prior to use. In the pre-harvest stage, Good Agriculture Practice (GAP) guidelines should be followed by produce growers to ensure the safety of water used for irrigation of fresh produce. In the post-harvest stage, Good Manufacturing Practices (GMPs) and Good Hygienic Practices (GHPs) guidelines recommended by the related agencies in each country need to be conformed to by food product manufacturers to assure that the products are of high quality and do not pose any risks to consumers. Different irrigation methods may pose different levels of risks associated with
produce contamination. Figure 1.1 shows the most and least risky methods for irrigating crops. Keeping irrigation water away from the edible parts of ready-to-eat crops results in a lowered risk of foodborne illness outbreak. Therefore, when choosing the most appropriate irrigation method for each specific crop, consideration should be given to the location of edible parts. Song et al. (2006) reported that subsurface drip method had a greater potential to reduce the health risks associated with the use of contaminated irrigation water than flood furrows in irrigating ground crops such as melons, lettuce, and bell peppers. Oron (2002) estimated that overhead sprinkler irrigation put the above ground produce at microbiological risks of foodborne disease outbreak 100 – 1000 times higher than drip irrigation method if the same water was used.

![Least Risky Irrigation Method](http://www.safeproduce.eu/WaterContact.htm)

Figure 1.1. The most and least risky water application methods for contaminated crops growing above ground with a little clearance (http://www.safeproduce.eu/WaterContact.htm).
Infected food handlers play an important role in the transmission of foodborne viruses in ready-to-eat foods and foods that are often handled by multiple people just before consumption. They can also contaminate the hard surface where viruses can survive for days or weeks, and then contaminate anything that touches those surfaces. The major concern is the asymptomatic infected handlers could contaminate foods unknowingly. Therefore, personal hygiene remains a key prevention strategy. GHPs and GMPs should be followed by the food industries to meet their food safety and quality responsibilities. Employees should be trained with the knowledge of foodborne illnesses. They should be trained with effective hand washing techniques. Effective hand-washing agents should be used. A mediated liquid soap containing 0.3% triclosan was found to be the most effective hand-washing agents to inactivate HAV and poliovirus on finger pads (Mbithi et al., 1993). If ineffective hand-washing agents are used, hand drying after washing is very critical because residual moisture on hand after drying promote virus transmission (D'Souza et al., 2007). Employees should be required not to work or handle food when displaying the symptoms such as vomiting, diarrhea, jaundice, and fever, etc. Employees should leave from work if they are exposed to foodborne pathogens by eating at a restaurant where an outbreak occurred or by living with someone who is diagnosed with certain foodborne illnesses. Effective cleaning and disinfecting surfaces are essential to eliminate viruses and to prevent them being introduced into food (National Disease Surveillance Centre, 2004).

Some viruses could be transmitted from animals to humans. The prevention strategies of foodborne zoonotic viruses need to be established. Animals shed viruses in their feces, and inadequate treatment of sewage containing animal feces may lead to the contamination of
irrigation water. The HEV in polluted irrigation water could be transmitted to fresh produce and then infect human. The HEV contaminated drinking water for animal feeding can cause animal infections. Consumption of raw or undercooked meat, or meat products from HEV-infected animals are capable of causing human hepatitis E. The infected meat needs to be boiled or stirred for 5 min to completely inactivate HEV (Feagins et al., 2008). Coastal waters may also be contaminated by HEV, resulting in the accumulation of the virus in shellfish. Thus, managing the sewage treatment and controlling the contamination of water in the early food chain is still the key measure for preventing zoonotic HEV illness. For Nipah virus, setting physical barriers that are able to prevent bats from contacting sap is a feasible measure to inhibit Nipah virus illness (Khan et al., 2012). Eggs can contain HPAI-H5N1 virus both on the outside shell and in the inside white and yolk, and so eggs from the area with H5N1 outbreaks should not be consumed raw or partially cooked and uncooked eggs should not be used in food products that will not be heat-treated. HPAI-H5N1 could survive at 4°C for more than 100 days and much longer time at < -20°C, and so low temperature is not a means of controlling the virus (Shabid et al., 2009; Shoham et al., 2012). Cooking temperature above 70°C will inactivate H5N1 virus (Thomas & Swayne, 2007). GAPs, such as supplying a source of potable water, providing a feed supply that is free of contaminants, and disinfection of the premises and equipment prior to the introduction of a new flock, need to be implemented to control infection of poultry and the transmission of disease among poultry on farm. When slaughtering poultry, GHP guidelines are essential for preventing exposure to the virus via raw poultry meat and cross contamination from poultry to other food or food preparation surfaces. Properly cooked poultry meat is reported to be safe for human consumption (Chmielewski & Swayne, 2011).
In addition to the aforementioned GAPs, GHPs, and GMPs, hazard analysis and critical control point (HACCP) programs have been required by the U.S. Food and Drug Administration (USFDA) and the U.S. Department of Agriculture (USDA) to be implemented mandatorily for seafood, juice and meat production (USFDA, 2001, 2011; USDA, 1999, 2005, 2006). The HACCP program aims at eliminating, preventing or reducing microbiological, chemical or physical hazards to an acceptable level during the process of food production rather than finished product inspection. The program is an effective approach for food safety. However, the program needs to be optimized for the foodborne viral prevention especially for the new emerging viruses.

Timely investigation and reporting of foodborne viral disease is of great importance for preventing the spread of infection, reducing severe economic loss and social impact on the industry. Reliable methods for the detection of viruses in food could facilitate the investigation of the food-related outbreaks by identifying the source quickly, implementation of control measures and evaluating the efficacy of intervention strategies. It is challenging to detect viruses in food because viruses are typically present at low levels in contaminated foods, and unlike bacterial pathogens, most viruses cannot readily be enriched by culture methods. Therefore, the standard methods used for bacterial detection are not suitable for viruses. The procedures for rapid detection of viruses in food are usually separated into three different stages: separation of viral particles from food matrices; concentration of the separated viral particles to a small volume; and detection of viral nucleic acid. No method is currently available for diagnosis of novel emerging viruses in food. Ideally, the detection method should be simple, sensitive, robust, and practical. In this thesis, HAV was used as a model virus for
developing the novel methods for the concentration and detection of the virus in different food matrices. The established methods could be adapted for the detection of other foodborne viruses, bacteria, chemicals, or toxins. The detailed information of the features of HAV, the resistance of HAV against food processing conditions, as well as the concentration and detection methods of HAV in foods are reviewed in the following parts. The separation, concentration and detection methods described in the following parts are not limited to HAV.

1.3. HAV

1.3.1. Characteristics of HAV

In 1973, the disease “catarrhal jaundice” was renamed “hepatitis A” by WHO, and since then, the etiological agent causing the disease has been termed HAV (Gust & Feinstone, 1988). HAV belongs to the genus of *Hepatovirus* and the family of *Picornaviridae*. It has a non-enveloped icosahedral capsid of 27 – 30 nm in diameter, and the capsid contains a positive-sense ssRNA molecule of approximate 7.5 kb (Koopmans & Duizer, 2004; Nainan et al., 2006). The RNA genome (Figure 1.2) contains a single open reading frame (ORF) and bears different regions: the 5’ and 3’ untranslated regions (UTRs) with the 5’ end covalently linked to a small viral protein genome-linked (VPg) protein and 3’ end followed by a polyadenylated tail; the P1 region encoding viral structural proteins VP1, VP2, VP3, and putative VP4; and the P2 and P3 regions encoding nonstructural proteins including a viral RNA helicase, 3C protease (3C\textsuperscript{pro}), and 3D polymerase (3D\textsuperscript{pol}, an RNA-dependent RNA polymerase (RdRp). These proteins are implicated into the replication of HAV infectious cycle inside host cell (Nainan et al., 2006; Pintó et al., 2010). By analyzing the nucleotide sequence of the putative VP1/2A junction region, HAV falls into six genotypes. An HAV genotype is defined as a
group of viruses with > 85% nucleotide sequence identity. The HAV genotypes I and III are further classified into subgenotypes (A and B) with sequence variability of < 7.5% (Pintó et al., 2010; Robertson et al., 1992). Genotypes I, II, and III are associated with human infections and genotypes IV, V and VI are of simian origin. Genotypes I and III are the most prevalent HAV genotypes isolated from humans. Subgenotype IA appears to be responsible for the majority of hepatitis A cases worldwide and subgenotype IB has been found in South America (Brazil and Peru), Africa, Australia, Europe (Sweden, Norway, Finland and Denmark), and Japan. HAV genotype IIIA has been shown to be responsible for human infection in Southeast and Central Asia (India, Nepal, Sri Lanka and Malaysia), the USA, and Europe (Sweden, Norway, the U.K., and Estonia). Only a few HAV cases are attributed to genotype IIIB (Costa-Mattioli et al., 2003; Stene-Johansen et al., 2005). All genotypes of HAV share one serotype, which makes it possible for lifelong immunity after HAV vaccination (Atreya, 2004; Jothikumar et al., 2005; Sánchez et al., 2007). HAV outbreaks have reduced in the past decade in many countries as a result of vaccination campaigns (Daniels et al., 2009; Pintó et al., 2010). However, novel antigenic HAV variants have been recently observed making vaccination ineffective. In Catalonia, Spain, the outbreaks of HAV increased during the period 2002 – 2009 in spite of annual vaccination campaigns (Pérez-Sautu et al., 2011a). Costa-Mattioli et al. (2002a) determined the nucleotide sequences of the complete VP1 gene of 81 strains isolated from serum samples of patients from France, Kosovo, Mexico, Argentina, Chile, and Uruguay, and through phylogenetic analysis of the sequences, they identified one novel antigenic HAV variant with a 15-amino-acid deletion located on the VP1 region, which contributed to the major immunodominant antigenic site of HAV. This strain might be the first antigenic variant of HAV found in humans. Pérez-Sautu et al. (2011b) isolated six HAV
antigenic variants that likely escaped the protective effect of available vaccines from patients infected with HAV in an outbreak in Catalonia, Spain, during 2005 and 2009. These antigenic variants showed amino acid replacements at positions 1166 – 1280 located at viral immunodominant sites in VP1 region. Sánchez et al. (2002) found two HAV antigenic variants from serum samples of patients with hepatitis A for the consumption of contaminated clams in Spain in 1999. One antigenic variant occurred in a discontinuous epitope defined by monoclonal antibody (MAb) K3-4C8 and the other in a linear VP1 epitope of the virus. The mutant variants showed 81% and 35% reductions in recognition by MAb K3-4C8 and K2-4F2, respectively. Gharbi-Khelifi et al. (2007) reported a discovery of two HAV antigenic variants in Tunisia. The variants, Tun31-03 and Tun36-03, were isolated from serum samples collected from 99 patients with hepatitis A. The Tun31-03 antigenic variant showed a 38-amino-acid (aa) deletion located between 150 and 1887 aa of the VP1 protein where neutralization escaped mutation. The Tun36-03 antigenic variant presented a substitution of threonine by proline at position 10 of the VP1 protein. This amino acid is located in a peptide presenting an antigenically reactive epitope of the VP1 protein.

![Figure 1.2. Schematic representation of HAV genome (Nainan et al., 2006; Pintó et al., 2010).](image)
1.3.2. HAV infection

HAV (10 – 100 particles) causes an acute and self-limiting infection through the fecal-oral route (Yezli & Otter, 2011). After ingestion, HAV replicates in the intestinal tract, spreads to the liver through the blood stream, and then invades hepatocytes (Sinclair et al., 2009). The incubation period of HAV infection can be 15 – 50 days, with an average of 28 days (Castrodale et al., 2002). The general symptoms of HAV infection include fever, malaise, headache, fatigue, nausea, abdominal discomfort, liver inflammation, jaundice, and dark-colored urine. These symptoms can last for 1 – 2 weeks or longer. Excretion of viral particles in stools starts before the appearance of symptoms and continues for at least 4 weeks after the cessation of symptoms, providing many opportunities for fecal contamination of foods and person-to-person transmission (Bosch et al., 2008; Hirneisen et al., 2010).

1.3.3. Foodborne outbreaks of HAV

HAV has been found in contaminated shellfish (clam, oyster, and mussel) (Coelho et al., 2003; Croci et al., 2003; Goswami et al., 2002), fresh produce (green onion, lettuce, tomato, fig, and date) (Boxman et al., 2012; Sun et al., 2012; Wheeler et al., 2005), ready-to-eat foods (sandwich, coleslaw, French fries, sushi, and deli meat), ice cream, yogurt (Sun et al., 2012), milk, beef burger (Zaher et al., 2008), and orange juice (Frank et al., 2007). Actually, the number of foodborne HAV outbreaks is always under-estimated, because the incubation period of HAV is very long and the implicated foods usually have been consumed or discarded before the appearance of symptoms (Nainan et al., 2006). In up to 50% of HAV infections, the source is not identified. Fewer than 5% of the infections are classified as
foodborne, but a greater percentage may be transmitted by this route (Le Guyader & Atmar, 2008). Some selected foodborne HAV outbreaks are summarized in Table 1.4.
Table 1.4. Selected foodborne HAV outbreaks.

<table>
<thead>
<tr>
<th>Year</th>
<th># of cases</th>
<th>Country</th>
<th>Food type</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>2013</td>
<td>165</td>
<td>USA</td>
<td>Pomegranate seeds</td>
<td>(Collier et al., 2014)</td>
</tr>
<tr>
<td>2013</td>
<td>800</td>
<td>Italy</td>
<td>Frozen berries</td>
<td><a href="http://barfblog.com/tags/europe/">http://barfblog.com/tags/europe/</a></td>
</tr>
<tr>
<td>2013</td>
<td>103</td>
<td>Denmark, Finland, Norway, and Sweden</td>
<td>Frozen strawberry</td>
<td>(Nordic outbreak investigation team, 2013)</td>
</tr>
<tr>
<td>2013</td>
<td>80</td>
<td>Denmark, England, Germany, the Netherlands, Norway, and Sweden</td>
<td>Unknown</td>
<td>(MacDonald et al., 2013)</td>
</tr>
<tr>
<td>2012</td>
<td>6</td>
<td>Canada</td>
<td>Pomegranate seeds</td>
<td>(Swinkel et al., 2014)</td>
</tr>
<tr>
<td>2011</td>
<td>27</td>
<td>Japan</td>
<td>Sushi</td>
<td>(Tominaga et al., 2012)</td>
</tr>
<tr>
<td>2011</td>
<td>9</td>
<td>England and the Netherlands</td>
<td>Semidried tomatoes</td>
<td>(Carvalho et al., 2012)</td>
</tr>
<tr>
<td>2010</td>
<td>59</td>
<td>France</td>
<td>Semidried tomatoes</td>
<td>(Gallot et al., 2011)</td>
</tr>
<tr>
<td>2009</td>
<td>144</td>
<td>Australia</td>
<td>Semidried tomatoes</td>
<td>(Donnan et al., 2012)</td>
</tr>
<tr>
<td>2008</td>
<td>21</td>
<td>Austria</td>
<td>Unknown</td>
<td>(Schmid et al., 2009)</td>
</tr>
<tr>
<td>2006</td>
<td>16</td>
<td>France</td>
<td>Liver pate</td>
<td>(Schwarz et al., 2008)</td>
</tr>
<tr>
<td>2005</td>
<td>39</td>
<td>USA</td>
<td>Oysters</td>
<td>(Bialek et al., 2007)</td>
</tr>
<tr>
<td>2005</td>
<td>16</td>
<td>Canada</td>
<td>Unknown</td>
<td>(Heywood et al., 2007)</td>
</tr>
<tr>
<td>2004</td>
<td>269</td>
<td>Belgium</td>
<td>Raw beef</td>
<td>(Robesyn et al., 2009)</td>
</tr>
<tr>
<td>2003</td>
<td>601</td>
<td>USA</td>
<td>Green onions</td>
<td>(Wheeler et al., 2005)</td>
</tr>
</tbody>
</table>

(Continued on next page)
<table>
<thead>
<tr>
<th>Year</th>
<th>Number</th>
<th>Country</th>
<th>Product</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>2002</td>
<td>26</td>
<td>Italy</td>
<td>Unknown</td>
<td>(Chironna et al., 2004)</td>
</tr>
<tr>
<td>2002</td>
<td>43</td>
<td>New Zealand</td>
<td>Blueberries</td>
<td>(Calder et al., 2003)</td>
</tr>
<tr>
<td>2001</td>
<td>43</td>
<td>USA</td>
<td>Sandwiches</td>
<td>(LaPorte et al, 2003)</td>
</tr>
<tr>
<td>1999</td>
<td>184</td>
<td>Spain</td>
<td>Clams</td>
<td>(Sánchez et al., 2002)</td>
</tr>
<tr>
<td>1998</td>
<td>43</td>
<td>USA</td>
<td>Green onions</td>
<td>(Dentinger et al., 2001)</td>
</tr>
<tr>
<td>1997</td>
<td>467</td>
<td>Australia</td>
<td>Oysters</td>
<td>(Conaty et al., 2000)</td>
</tr>
<tr>
<td>1997</td>
<td>213</td>
<td>USA</td>
<td>Frozen strawberries</td>
<td>(Hutin et al., 1999)</td>
</tr>
<tr>
<td>1994</td>
<td>16</td>
<td>Italy</td>
<td>Clams</td>
<td>(Leoni et al., 1998)</td>
</tr>
<tr>
<td>1990</td>
<td>28</td>
<td>USA</td>
<td>Frozen strawberries</td>
<td>(Niu et al., 1992)</td>
</tr>
<tr>
<td>1988</td>
<td>292,301</td>
<td>China</td>
<td>Clams</td>
<td>(Halliday et al., 1991)</td>
</tr>
<tr>
<td>1988</td>
<td>61</td>
<td>USA</td>
<td>Oysters</td>
<td>(Desenclos et al., 1991)</td>
</tr>
</tbody>
</table>
1.3.4. Stability of HAV

Although not able to replicate outside of the host, HAV can remain infectious on environmental surfaces or in food products for a substantial period of time. The survival of HAV under different environmental or food processing conditions is described in the following parts.

1.3.4.1. Survival of HAV on animate and inanimate surfaces

A study by Mbithi et al. (1992) demonstrated that HAV could survive and remain infectious on human hands for more than 4 h and could be transferred between hands and inanimate surfaces. Virus-containing fecal material could adhere to fingernails, especially long and artificial fingernails, and transmit the spread of HAV (D'Souza et al., 2007). In another study by Mbithi et al. (1991), it was reported that HAV could survive on surface of stainless steel even longer than on human skin over a wide range of temperature and relative humidity, and the survival of the virus was reversely proportional to the temperature and relative humidity. The virus survived better at 5°C than at 20°C or 35°C. At 35°C and 95% relative humidity, the virus was undetectable after 4 h, whereas at 5°C and 95% relative humidity, 50% of infectious virus particle remained infectious. Abad et al. (1994) evaluated the survival of HAV, rotavirus, adenovirus, and poliovirus on several porous (paper and cloth) and nonporous (aluminium, china, glazed tile, polystyrene, and latex) materials. It was found that HAV and rotavirus survived significantly longer than adenovirus and poliovirus on all surfaces. HAV and rotavirus were shown to survive over 60 days on each surface, whereas the survival of poliovirus and adenovirus was seldom recorded beyond 30 days, although on paper they did survive up to 60 days. It was shown that, on non-porous surfaces, HAV was most resistant to
dessication, followed by rotavirus, adenovirus, and poliovirus. On porous materials, rotavirus was the most stable, followed by HAV, poliovirus, and adenovirus. Kim et al. (2012) evaluated the persistence of HAV on the surface of chopping boards made of wood or stainless steel under different temperatures (15, 25, 32, and 40°C) and found HAV survived better at lower temperatures, which was in good agreement with the previous report (Mbithi et al., 1991). The researchers also found that the virus survived longer on wood than on steel. A study by Butot et al. (2007b) demonstrated that enteric viruses, such as HAV, NoV, and rotavirus, could adhere to the wall of polyethylene terephthalate (PET) bottles used for water storage. After 20 days of water storage, the retention levels of HAV, NoV, and rotavirus on the wall of PET bottles were 90%, 85%, and 80%, respectively. The viruses might be absorbed on the wall of bottles through electrostatic force, hydrogen bonding, van der Waals forces, or hydrophobic interactions. These results indicate that bottle analysis is of great significance to evaluate the viral contamination of water.

1.3.4.2. Persistence of HAV during food storage

HAV can survive at 2 – 8°C for a period of time exceeding the shelf life of products. Croci et al. (2002) studied the survival of HAV on lettuce stored at 4°C for 9 days. The virus remained vital until the last day of storage and only a 2-log_{10} decrease of the quantity of virus was detected. Hewitt & Greening (2004) evaluated the persistence of HAV and NoV in marinated mussels over 4 weeks at 4°C and found there were no reduction in the titer of both viruses. Shieh et al. (2009) reported that HAV was able to survive on spinach leaves in moisture- and gas-permeable packages stored at 4°C for 7 weeks with slightly > 1-log_{10} reduction of infectivity. Sun et al. (2012) studied HAV inactivation rates in contaminated green onions
contained in gas-permeable, moisture-retaining high-density polyethylene packages that were stored at 3, 10, 14, 20, 21, 22, and 23°C, and found that HAV could survive in green onions for 29 days when the samples were stored at 4°C, and increases in the storage temperature resulted in greater inactivation rates of HAV, with 1°C increase in temperature would enhance inactivation of HAV by 0.007 log$_{10}$ plaque-forming unit (PFU)/day in green onions. Biziagos et al. (1988) compared the persistence of HAV and poliovirus in mineral water stored at 4°C and room temperature and observed that both viruses could keep infectivity at 4°C for 1 year, and poliovirus was not detected whereas HAV was still infectious after storage at room temperature for 300 days. Freezing did not significantly reduce the viability of HAV and rotavirus on fresh produce such as blueberries, strawberries, raspberries, basil, and parsley stored at -20°C for 3 months (Butot et al., 2008). Wang et al. (2013) investigated the persistence of HAV on alfalfa seeds during storage at 22°C, and found that the virus remained infectious after 50 days. Following storage, the alfalfa seeds were germinated in sprout growth chambers for 7 days, and the presence of HAV was found in all tissues of the sprouts as well as in the sprout-spent water.

1.3.4.3. Resistance of HAV to chemical disinfectants

HAV is stable to acid treatment and demonstrates infectivity following exposure to a pH of 1.0 for 5 h at 24°C (Scholz et al., 1989). Hewitt & Greening (2004) inoculated HAV to acid marinade at pH 3.75, and found that HAV still survived after 4 weeks. The morphology and physiology of food surfaces can affect the effectiveness of chemical disinfectants. The rough and ridged leaves make decontamination of leafy greens challenging, and porous and uneven surfaces of soft fruits like blackberries and raspberries make viruses entrapped (D'Souza et al.,
Butot et al. (2008) simulated the post-harvest rinsing conditions with chlorine, the most widely used sanitizing agent for fresh produce, to remove and inactivate enteric viruses (HAV, NoV, and rotavirus) on blueberries, strawberries, raspberries, basil, and parsley. They found that the chlorine caused $< 2.4\log_{10}$ reduction of HAV in all tested items, $< 3\log_{10}$ reduction of rotavirus in blueberry, basil, and parsley, and $\leq 3\log_{10}$ reduction of NoV GII in all samples. Fraisse et al. (2011) found that a simple washing of lettuce by water only resulted in an approximate $0.7\log_{10}$ reduction of HAV. And with the addition of 15 ppm of chlorine in water, a $1.9\log_{10}$ reduction of HAV could be obtained. Whereas, 100 ppm of peroxyacetic-based biocide only resulted in a $0.7\log_{10}$ reduction of HAV on lettuce. Jean et al. (2003) evaluated the effects of three commercial disinfectants, 2.9% dodecylbenzene sulfonic acid plus 16% phosphoric acid, 10% quaternary ammonium, and 2% iodide, commonly used in the food industry, on the inactivation of HAV in solution, and found that these disinfectants could not reduce HAV by $> 2.5 \log_{10}$ even at very high concentrations. The researchers also evaluated the effects of three other commercial disinfectants, 10% quaternary ammonium plus 5% glutaraldehyde, 12% sodium hypochlorite, and 2% stabilized chlorine dioxide, on the inactivation of HAV attached to agri-food surfaces, and found that chlorine dioxide was not able to inactive HAV effectively ($< 2.5 \log_{10}$-reduction) and for the other two disinfectants, the reduction rate of HAV could be improved to be $\geq 3 \log_{10}$ by increasing the contact time, the concentration of the disinfectants and working temperature. Gerba & Kennedy (2007) found that solely using the detergent in common use in laundering practices in the U.S. could not effectively remove or inactivate HAV, rotavirus and adenovirus on clothes, but when used together with sodium hypochlorite in laundry, the detergent could reduce the enteric viruses effectively by at least 99.99%. Sabbah et al. (2010) used an environmental surface
disinfectant, peracetic acid (1,000 ppm), to treat a mixture of *Acinetobacter baumannii*, *Mycobacterium terrae*, HAV, and spores of *Geobacillus stearothermophilus*, and found that the disinfectant was effective against the two bacteria and the spores (≥ 6-log_{10} reduction) but not effectively against the virus (≤ 3-log_{10} reduction). Poliovirus, coxsackievirus and HAV were more resistant to HClO than coliform or enteric pathogenic bacteria (Pirtle & Beran, 1991). Current chemical decontamination strategies are not very efficacious, and the more efficacious chemical disinfection methods for HAV on foods need to be developed (D'Souza et al., 2011).

### 1.3.4.4. Resistance of HAV to thermal processing

Traditionally, thermal processes have been used extensively in food technology for inactivation of foodborne pathogens (van Boekel et al., 2010). HAV has been found to be resistant to temperatures up to 60°C. Emerson et al. (2005) compared the thermal stability of HAV and HEV through heating the fecal suspensions of the viruses to temperatures between 45°C and 70°C for 1 h, and residual infectivity of the viruses were determined by a cell culture system. It was found that only 50% of HAV was inactivated by incubation at 60°C for 1 h and HAV was almost totally inactivated by incubation at 66°C for 1 h. For HEV, the 50% inactivation temperature was between 45°C and 50°C, and almost the entire virus was inactivated at 56°C. HAV in contaminated mussels could survive during cooking. Croci et al. (2005) investigated the resistance of HAV in mussels subjected to three different traditional Italian cooking methods: mussels were mixed with water and put in a covered container and cooked on a gas stove for 9 min; the opened mussels were covered with a mixture of onions, garlic, parsley, breadcrumbs, and butter, and then grilled in the oven (250°C) for 5 min; oil,
garlic, tomato sauce and water were cooked in a pot for 15 min, then mussel bodies, oregano and parsley were added and cooked again for 8 min at boiling temperature. The results showed that the infectious viruses were completely inactivated only in the “mussel in tomato sauce” method, while they were still infective in the other preparations. Hewitt & Greening (2006) demonstrated that when boiled in water for 170 s, the internal temperature of the New Zealand Greenshell mussel (Perna canaliculus) arrived at 90°C, and with continuous boiling for 40 s, the mussel shell was opened. When steaming was used, the mussel shell opened after the internal temperature achieved at 83°C for 300 s. However, it was reported that the conditions for complete inactivation of viruses in soft-shell clams (Mya arenaria) were maintaining the internal temperature at 90°C for 180 s (Sow et al., 2011). In another study, a heat treatment of 10 min at 90°C was required to inactivate HAV in infected clams (Ruditapes philippinarum) (Cappellozza et al., 2012). Thus, cooking shellfish until the shell just opened was not enough to inactivate viruses inside. Steaming mussels for less than 6 min was not sufficient to inactivate HAV (Harlow et al., 2011). In order to make the thermal inactivation behavior of HAV in blue mussels (Mytilus edulis) clear and give proper directions to shellfish processing industry, Bozkurt et al. (2014) studied the thermal inactivation kinetics of HAV in blue mussels and found that the relationship between viral reduction and time-temperature treatment was nonlinear. Two models, Weibull model and first-order model, were used to analyze experimental data. The Weibull model was more appropriate to represent the thermal inactivation behavior of HAV in blue mussels than the first-order model. Using the Weibull model, the D-values (50 – 72°C) ranged from 1.57 to 37.91 min for HAV. At 72°C, the required treatment time to achieve a 6-log reduction was 8.47 min. The z-values calculated for HAV were 15.88 ± 3.97°C ($R^2 = 0.94$). The calculated activation energies were 153 kJ/mole.
Hewitt et al. (2009) investigated the effect of heating at 63°C or 72°C for 10 min on the inactivation of HAV in milk and water. It was found that after exposure at 63°C for more than 5 min in water and milk, HAV showed reductions of infectivity titer of $> 3.5 \log_{10}$. When exposed at 72°C for 2 min in milk and 1 min in water, the virus showed at least a 3.5-$\log_{10}$ reduction. Butot et al. (2009) reported that enteric viruses in contaminated herbs could be effectively inactivated by steam blanching at 95°C for 2.5 min. HAV on contaminated green onions could be reduced by $> 3 \log_{10}$ during low heat dehydration at 65°C for 20 h (Laird et al., 2011).

Food constituents and physiochemical factors can affect the viral sensitivity to thermal treatments. Deboosere et al. (2004) investigated the inactivation of HAV in strawberry puree and found that increasing sucrose concentrations could result in a protective effect of HAV at 85°C. Deboosere et al. (2010) investigated the effect of heat treatments between 65°C and 75°C on survival of HAV in acidified red berries with pH between 2.5 and 3.5. It was found that the reduction in viral titer was greater for higher temperatures and lower pH and the influence of pH was more pronounced at higher temperatures. Higher fat content also increased the heat resistance of HAV, since cream with higher fat content required longer exposure to heat to achieve the same level of reduction of HAV as skim milk with lower fat content (Bidawid et al., 2000a).

1.3.4.5. Persistence of HAV against depuration process in shellfish

Filter-feeding shellfish can bioaccumulate pathogenic microorganisms in up to 1000-fold levels higher than those found in the surrounding environment (Love et al., 2010). To control
shellfish-borne diseases, a commercial treatment process, depuration, has been used. Depuration means immersing the contaminated shellfish in tanks of clean seawater to allow the contaminants to be purged (Lees et al., 2010). This process works more efficiently for eliminating bacteria from shellfish than for viruses. For example, oysters eliminated only ~7% of Norwalk virus compared to 95% reduction in levels of *E. coli* over a 48 h depuration period (Schwab et al., 1998). In a study by Love et al. (2010), oysters (*Crassostrea virginica*) and hard shell clams (*Mercinaria mercinaria*) were artificially contaminated with *E. coli, Enterococcus faecalis*, poliovirus, and HAV, and then depurated in 5-day trials with daily sampling. It was found that in oysters, depuration rates from the greatest to least were: *E. coli* > *Enterococcus faecalis* > poliovirus > HAV. In clams, depuration rates from the greatest to least were: *E. coli* > *Enterococcus faecalis* > HAV > poliovirus. Thus, fecal bacteria appear to be poor process indicators of the virological quality of the depurated oysters and hard shell clams. Mcleod et al. (2009) investigated the removal of HAV, NoV and poliovirus from Pacific oysters (*Crassostrea gigas*) by depuration and found that after cleansing for 23 h, a large drop of count in poliovirus was identified, whereas the levels of HAV and NoV did not decrease significantly. In Manila clams, HAV levels remained relatively high and viral particles were still infectious after depuration for 7 days (Polo et al., 2014). After depuration against pathogen-free seawater for one month, HAV and NoV were still detected in the contaminated oysters (*Crassostrea ariakensis*) (Nappier et al., 2008). It was reported that enteric viruses, such as HAV, poliovirus and murine NoV, could bind to hemocytes within oysters, which played an important role in the retention of viral particles (Provost et al., 2011).
1.3.4.6. Stability of HAV under non-thermal food processing conditions

The non-thermal food processing techniques that have been commercially applied in food industries include high hydrostatic pressure (HHP), ozone treatment, ultraviolet (UV) treatment, and electron beam irradiation, etc. (van Boekel et al., 2010; Hirneisen & Kniel, 2013; Praveen et al., 2013). In comparison with thermal processing, some of these non-thermal techniques can allow the retention of fresh-like organoleptic properties of food. Corrêa et al. (2012) applied UV treatment to disinfect HAV in seawater using a 36 W lamp and observed a 3.5-log$_{10}$ reduction of HAV after 120 h treatment. The UV treatment could be applied to disinfect water used for depuration of shellfish. However, in a study by Diego et al. (2013), it was reported that even with UV treatment of depuration tanks, HAV and NoV were still detectable in oysters.

A series of studies has been performed by the same research group using HHP to inactivate enteric viruses in different matrices. In a study, a HAV stock with a titer of 7 log$_{10}$ PFU/mL was reduced to non-detectable levels after exposure to HHP at 450 megapascals (MPa) for 5 min, whereas poliovirus was unaffected by a 5-min treatment at 600 MPa. It was also found that the pressure resistance of HAV was increased in seawater, indicating that salts had a protective effect on virus inactivation (Kingsley et al., 2002). Increasing the treatment temperature and decreasing the solution pH could enhance pressure inactivation of HAV in solutions. However, increasing the treatment temperature did not have any significant effect on the inactivation of HAV in oyster homogenates (Kingsley & Chen, 2009). There was no significant difference between inactivation of HAV in whole-in-shell oysters and shucked oysters after a 400-MPa HHP treatment. For whole-in-shell and shucked oysters, an average
2.56-log_{10} and 2.96-log_{10} reduction of HAV was obtained, respectively (Kingsley et al., 2009). For using HHP to inactivate HAV within mussels, similar results to oysters were obtained by Terio et al. (2010). The researchers found that a treatment performed with a hydrostatic pressure of 400 MPa for 5 min at room temperature resulted in a 3.6-log_{10} reduction of HAV in blue mussels (*Mytilus edulis*) and a 2.9-log_{10} reduction of the virus in Mediterranean mussels (*Mytilus galloprovincialis*). In another study, a reduction of 4.32 log_{10} of HAV in strawberry puree and 4.75 log_{10} in sliced green onions could be obtained when the samples were exposed to a pressure of 375 MPa for 5 min at room temperature. The inactivation efficiency of HAV decreased with the declining of treatment pressures (Kingsley et al., 2005). HHP was also used for inactivation of HAV in pork sausage products, and a reduction of 3.23 log_{10} 50% tissue culture infective dose (TCID_{50})/mL was obtained after treatment with a pressure of 500 MPa for 5 min at 4°C (Sharma et al., 2008).

Hirneisen & Kniel (2013) evaluated the effects of four processing technologies, UV (240 mJ/s/cm^{2}), ozone (6.25 ppm for 10 min), high pressure (500 MPa for 5 min at 20°C), or calcium hypochlorite spray (150 ppm, 4°C), on inactivation of HAV in/on green onions. For inactivation of HAV on surface of the green onions, high pressure performed the most efficiently with a 5.5-log_{10} reduction of HAV, followed by UV, ozone, and calcium hypochlorite treatment, making a reduction of 5.2-log_{10}, 2.9-log_{10}, and 2.6-log_{10} of HAV, respectively. For inactivation of HAV of the interior of the green onion, high pressure still led to the highest reduction of the virus, followed by ozone, UV, and calcium hypochlorite treatment, resulting in a reduction of 2.5-log_{10}, 1.5-log_{10}, 0.4-log_{10}, and 0.4-log_{10} of HAV, respectively. Jean et al. (2011) used pulsed UV light to inactivate HAV and a NoV research
surrogate, murine norovirus-1 (MNV-1), in liquid suspension and on surfaces of stainless steel and polyvinyl chloride disks. The researchers found that the pulsed UV light performed well on inactivation of viruses on inert surfaces or in suspensions with a reduction of viruses at least 3 log_{10} units. They also found that the inactivation efficiency of viruses was higher on inert surfaces than in suspension. Praveen et al. (2013) investigated the inactivation of HAV and MNV-1 in oysters (*Crassostrea virginica*) by electron beam irradiation. With the employment of maximum FDA-allowable doses (5.5 kGy) of electron beam irradiation, the reduction of HAV and MNV-1 in whole oysters did not exceed 94% and 90%, respectively.

1.4. Separation and concentration methods of foodborne viruses

HAV particles may be found in large numbers in stool (≥ 10^6 virus particles per gram), but they are usually present in low numbers in foods (Cliver, 1997). So far, there is not much information regarding the level of HAV contamination in food products. The reported contamination level is 0.2–224 particles/100 g of shellfish meat (Williams & Fout, 1992), 10^3 – 10^5 HAV genomes/g of clam (Atmar et al., 1993; Lees, 2000), 1.1 × 10^2 to 4.1 × 10^6 HAV genomes/g digestive tissue of mussel (Manso & Romalde, 2013), 15 HAV genomes/30 g of dates (Boxman et al., 2012), or 2.4 × 10^2 to 2.4 × 10^3 HAV genomes/g of vegetables such as cilantro, parsley, and green onions (Felix-Valenzuela et al., 2012). Because of the low contamination level of HAV in food samples, the viral particles need to be separated and concentrated prior to detection. The overall sensitivity of the method is determined by the efficacy of each step.
1.4.1. Methods of foodborne virus separation

Viruses can be transmitted by food because viral particles are able to bind or attach on the surface of food and can survive for a specific period until the food is consumed. The interactions between food and viruses include mechanical entrapment, ionic bonding, van der Waals forces, electrostatic forces, and hydrophobic interactions (Le Guyader & Atmar, 2008). These interactions need to be broken in order to separate viruses from food. The complex surface of many fruits and vegetables may prevent removal of viruses from them by simple washing. For example, for removing HAV experimentally contaminated on the surface of lettuce, fennel, and carrot, the simple washing by water did not reduce virus titer substantially (Croci et al., 2002). Thus, a buffer with a given pH and ionic strength should be used to break the linkage between virus and food. These include using 3% beef extract (pH 9.5) to elute HAV from lettuce, carrot and fennel (Croci et al., 2002), using glycine-Tris buffer (pH 9.5, 50 mM glycine, 100 mM Tris) containing 1% – 3% beef extract to elute HAV, NoV, rotavirus and poliovirus from various berry fruits (raspberries and mixtures of raspberry, bilberry, blackberry, currant, blackcurrant, and cherry) and vegetables (lettuce, radishes, tomatoes, green onions, mint, parsley and basil) (Dubois et al., 2002; Butot et al., 2007a), using glycine buffer (pH 9.0, 50 mM glycine, 0.14 N salt) to elute HAV and NoV from lettuce and hamburger (Sair et al., 2002), using glycine buffer (0.25 N glycine, pH 10) to elute HAV from oyster or clam tissues (Casas et al., 2007; Suñén et al., 2004), using glycine buffer (0.05 M glycine, 0.15 M NaCl, pH 9.0) to elute NoV from shellfish (Baert et al., 2007), using glycine buffer (0.5 M glycine, 0.14 NaCl, 0.2% (v/v) Tween 20, pH 8.5) to elute HAV and NoV from lettuce and turkey (Jean et al., 2004), using 1 M sodium bicarbonate containing 1% (w/v) soya protein powder to elute NoV from raspberries (Rzeżutka et al., 2005), using phosphate-
buffered saline (PBS, pH 7.6) to elute HAV from lettuce and strawberries (Bidawid et al., 2000b), and using 2.9% (w/v) tryptose phosphate broth with 6% (w/v) glycine at pH 9.5 to elute HAV and NoV in green onions (Guévremont et al., 2006). The glycine buffer (0.05 M glycine, 0.14 M NaCl, 0.2% (v/v) Tween 20, pH 9.0) has been used by Health Canada in the Compendium of Analytical Methods for eluting HAV from strawberries (Mattison et al., 2010). Repeated pipetting/washing the surface of food with a buffer or gently shaking the food in a buffer are the methods currently used for eluting viral particles from food surface (Bidawid et al., 2000b; Croci et al., 2002; Guévremont et al., 2006).

1.4.2. Methods of foodborne virus concentration

The concentration step is necessary to achieve reduction in sample volume with recovery of virus and elimination of ingredients of food matrices. Currently used concentration methods include polyethylene glycol (PEG) precipitation, ultracentrifugation, ultrafiltration, filtration with charged membranes, and immunomagnetic separation (Stals et al., 2012).

1.4.2.1. PEG precipitation

PEG is a non-toxic water-soluble synthetic polymer and has been widely used as a precipitating agent for the purification of proteins. The mechanism of PEG precipitation is that PEG molecules in solution could attract water molecules away from the solvation layer around the protein, making proteins sterically excluded from regions of the solvent, thus the protein-protein interactions are increased and precipitation occurs (Atha & Ingham, 1981). The PEG precipitation method has been reported in numerous studies for the concentration of foodborne viruses. Kingsley & Richards (2001) used 16% (w/v) PEG 8,000 with 0.525 M NaCl to precipitate HAV and NoV from frozen hard-shell clams (Mercenaria mercenaria) and Eastern
oysters (*Crassostrea virginica*) by mixing the equal volume of the shellfish extracts and PEG solution together. The precipitation was performed on ice for 1 h followed by centrifugation at $10,000 \times g$ for 5 min at 4°C. Viral RNAs were purified using Tri-reagent and Dynabeads-oligo(dT)$_{25}$ and detected by reverse transcription-polymerase chain reaction (RT-PCR). The detection limits of HAV in clams and oysters were 0.15 PFU/3.75 g of tissue and 1.5 PFU/3.75 g of tissue, respectively. For NoV, the detection limit was 224 RT-PCR units in oysters. Dubois et al. (2002) added 10% (w/v) PEG 6,000 and 0.3 M NaCl to the neutralized eluates from fruits and vegetables contaminated with HAV, NoV, and poliovirus. The mixture was incubated at 4°C overnight and viruses were concentrated by centrifugation at $10,000 \times g$ for 2 h at 4°C. Viral RNAs were purified from the concentrates using the RNeasy Mini Kit (Qiagen, Courtaboeuf, France). When coupled with RT-PCR for detection, 50 TCID$_{50}$/100 g of HAV and poliovirus and 1200 RT-PCR units of NoV were detectable on products. Baert et al. (2007) used the PEG precipitation method to concentrate NoV in alkaline eluates from mussels. The neutralized eluates were incubated with 6% (w/v) PEG 6,000 and 0.3 M NaCl overnight. Then viral particles were concentrated by centrifugation at $10,000 \times g$ for 30 min at 4°C. The extracted viral RNAs by the RNeasy Mini Kit (Qiagen, Hilden, Germany) were amplified by real-time RT-PCR and 100 RT-PCR units of NoV were detectable. In a study by Guévremont et al. (2006), a PEG precipitation procedure was employed to concentrate HAV and NoV in green onion eluates by adding 16% (w/v) PEG 8,000 and 0.525 M NaCl to the eluates to achieve a final concentration of 8% (w/v) PEG. The viral suspensions were stored overnight at 4°C and the viral particles were collected by centrifugation at $12,000 \times g$ for 30 min at 4°C. The extracted RNAs by Trizol–chloroform and poly(dT) magnetic beads methods were amplified by RT-PCR. In 25 g of green onions, 1 TCID$_{50}$ of HAV and 1 RT-PCR unit of
NoV were detectable. Rutjes et al. (2006) reported that a PEG precipitation protocol demonstrated good efficiency in concentrating canine calicivirus in whipped cream. The treated cream was mixed with PEG 6,000-NaCl solution to make a final concentration of 10% (w/v) PEG-0.3 M NaCl. The mixture was rotated at 4°C for 2 h and then centrifuged for 30 min at 9,500 × g at 4°C to pellet viral particles. Viral RNAs were extracted with TRIzol and detected by RT-PCR. The results showed that 10% of the virus could be recovered from the cream. In a study by Lee et al. (2012), a PEG precipitation method was used to concentrate HAV from the wash-off of lettuce and perilla leaves. The virus was first precipitated using 40% (w/v) PEG 8,000 and 3 M NaCl for 16 h at 4°C followed by centrifugation at 16,000 × g for 20 min at 4°C to pellet the viral particles. The particles were re-suspended in 5 mL of supernatant and precipitated for the second time with the PEG precipitation method. Viral RNAs extracted using the QIAamp viral RNA Mini kit (Qiagen, Hilden, Germany) was tested by real-time RT-PCR. The recovery rate of HAV from lettuce and perilla leaves was 5.7% and 5.6%, receptively. The PEG precipitation method was also used to co-concentrate HAV, NoV, E. coli O157:H7, L. monocytogenes, and Salmonella from 10 g of fresh-cut vegetable products, including parsley, spinach, and salad. The vegetable eluate (95 mL) was supplemented with a final concentration of 10% (w/v) PEG 8,000 and 0.3 M NaCl. The samples were gently shaken for 1 h at 4°C and then centrifuged for 30 min at 10,000 × g at 4°C to obtain the pellet. The pellet was resuspended in PBS. Viral RNAs were extracted from 150 µL of pellet suspension using NucleoSpin® RNA virus kit (Macherey-Nagel GmbH & Co., Duren, Germany). Bacterial DNAs were extracted from 150 µL of pellet suspension using NucleoSpin tissue kit (Macherey-Nagel GmbH & Co., Duren, Germany). Both viral RNA and bacterial DNA were tested by real-time RT-PCR. The detection limits were 132 RT-PCR units, 6.6 TCID₅₀, 10³,
10^2, and 10^3 colony-forming unit (CFU)/g for NoV, HAV, *E. coli* O157:H7, *L. monocytogenes*, and *Salmonella*, respectively (Sánchez et al., 2012). For concentrating HAV from the rinse fluid of raspberries, the neutralized rinse fluid was supplemented with 10% (w/v) PEG 6,000 and 0.3 M NaCl to precipitate HAV for 2 h at 4°C. The viral particles were concentrated by centrifugation at 10,000 × g for 30 min at 4 °C and the pellet was re-suspended in 500 µL of PBS for RNA extraction using NucliSens® easyMAG platform (BioMérieux, Marcy l’Etoile, France). The limit of detection of HAV tested by real-time RT-PCR was 50 PFU/25 g of fresh raspberries and 100 PFU/25 g of frozen raspberries (Blaise-Boisseau et al., 2010). With the same protocol used to concentrate NoV from strawberries (5 g) and lettuce (25 g), Cheong et al. (2009) found that 50% of NoV could be recovered from strawberries and 2.9% of the virus could be recovered from lettuce. Different incubation time of PEG precipitation was reported in the above-mentioned studies. Jones & Johns (2009) used the PEG precipitation method to concentrate F-RNA coliphage MS2, a bacteriophage used as a surrogate for studying human enteric viruses like NoV and HAV, from fecal materials and found that the precipitation time of 2 h was not enough for obtaining the phage pellet. The highest recovery of the coliphage MS2 was obtained in the presence of 10% (w/v) PEG and 1 M NaCl after 16 h of precipitation.

### 1.4.2.2. Ultracentrifugation

Ultracentrifugation is a technique that is designed to spin rotors at high angular velocities, to generate a very high centrifugal speed forces up to 2,000,000 × g. This high force causes sedimentation of molecules with small size and low density, such as cellular organelles and viruses (http://en.wikipedia.org/wiki/Ultracentrifuge). This technique has been reported in the concentration of viruses in contaminated food and water. Rutjes et al., (2006) reported that
ultracentrifugation at 120,000 × g for 3 h worked efficiently for recovering canine calicivirus from the rinse fluid (9 mL) of lettuce (5 g), a food item of solid matrix, which produced a clear eluate after the virus was washed off from the food item. The method did not work well for whipped cream because excessive fat components accumulated on top of the supernatant, making it more difficult to remove the supernatant. The viral RNA extracted using RNeasy Mini Kit (Qiagen, Hilden, UK) was tested by real-time RT-PCR. The results showed that for an inoculum level of 10⁵ TCID₅₀ of canine calicivirus on lettuce, 10⁴ TCID₅₀ of the virus could be recovered by the ultracentrifugation protocol. An ultracentrifugation at 235,000 × g for 2 h was used by Rzeutka et al. (2006) for the concentration of HAV from the eluates from strawberries (100 mL) and raspberries (120 mL). QIAamp Viral RNA Mini kit (Qiagen, Hilden, UK) was used to extract RNA from the pellet and the RNA was tested by nested RT-PCR, giving a limit of detection of HAV 10⁴ RT-PCR unit/90 g of strawberries and 10³ RT-PCR unit/60 g of fresh raspberries. Casas et al. (2007) used the ultracentrifugation method to recover HAV from 50 mL of eluate from artificially contaminated oysters (25 g). The virus in the oyster extract was concentrated by ultracentrifugation at 100,000 × g for 1 h at 4 °C. The viral RNA was extracted using Total Quick RNA isolation kit (Talent™, Italy) and evaluated by real-time RT-PCR. The limit of detection was 25 TCID₅₀ of HAV/25 g of oysters. Kovač et al. (2009) found that the ultracentrifugation method was able to improve the recovery efficiency of HAV in treated bottled water samples (15 mL) by approximately 20% in comparison with ultrafiltration method. Martin-Latil et al. (2012) used 10% (w/v) PEG 6,000 and 0.3 M NaCl to precipitate HAV from 40 mL of rinse fluid from semi-dried tomatoes and suspended the viral pellet in 10 mL of water, and then used ultracentrifugation to concentrate the virus at 100,000 × g for 1.5 h. The viral RNA was extracted using NucliSens® easyMAG
platform (BioMérieux) and tested by real-time RT-PCR. The detection limit of HAV given by the combined protocol was 1 PFU/25 g of sample.

1.4.2.3. Ultrafiltration

Ultrafiltration is a membrane filtration technique in which forces like pressure or concentration gradients lead to a separation through a semipermeable membrane with a specific molecular weight cut-off (MWCO). The suspended solids with high molecular weight are retained in the retentate while water and low molecular weight suspended solids pass through the membrane (http://en.wikipedia.org/wiki/Ultrafiltration). There are some reports using this technique to concentrate viruses from water or rinse solution of food. Soule et al. (2000) used an ultrafiltration system, the Minitan® system (Millipore) to concentrate poliovirus, rotavirus, and HAV from 2 L of water. The initial sample could be concentrated to approximately 15 mL, and was then concentrated to about 0.5 mL using Centriprep® concentrator (Amicon). After two steps of ultrafiltration, the three viruses were simultaneously recovered and tested by RT-PCR, allowing $10^3$ TCID$_{50}$ of poliovirus, 1 TCID$_{50}$ of rotavirus and 1 TCID$_{50}$ of HAV detectable in 1 L of water. Vaidya et al. (2004) reported an application of Amicon cell-based ultrafiltration unit to concentrate HAV from 10 mL of experimentally contaminated water. Polyacrylonitrile membrane with a MWCO of 60 – 70 kDa was used in the unit. The HAV in water with a concentration of $1.9 \times 10^4$ virions/mL could be detected using this method. Rutjes et al. (2006) used a Centricon Plus-20 filter (Millipore, Etten-Leur, The Netherlands) to concentrate 10 mL of food eluate containing canine calicivirus to 100 – 200 µL. The viral RNA was extracted from the concentrated sample using the RNeasy Mini Kit (Qiagen, Hilden, Germany). The researchers found that for $2.5 \times 10^5$ TCID$_{50}$ of inputted
virus, 1% of the virus could be recovered from contaminated lettuce, and for $2.5 \times 10^6 \text{TCID}_{50}$ of inputted virus, 0.1% of the viruses could be obtained from whipped cream. Butot et al. (2007a) used a Centricon Plus-70 centrifugal filter device (100K norminal molecular weight limit; Millipore, Molsheim, France) to concentrate HAV, NoV, and rotavirus from 60 mL of eluates from berry fruits and vegetables to about 600 µL. Viral RNAs were quantified by real-time RT-PCR and the detection limits of HAV, NoV, and rotavirus were 1 TCID$_{50}$, 54 RT-PCR units, and 0.02 TCID$_{50}$ per 15 g of food, respectively. The researchers also compared the efficiency of a PEG precipitation method and the ultrafiltration method for the concentration of HAV, NoV, and rotavirus from strawberries and raspberries, and found that the ultrafiltration method showed higher viral recovery rates than the PEG precipitation method. Summa et al. (2012) used an ultrafiltration protocol to concentrate NoVs in eluates from fresh lettuce, sliced ham, and frozen raspberries. The ultrafiltration was performed with a microconcentrator tube Amicon Ultra-15 Centrifugal Filter Device (Millipore, USA) to concentrate the virus from 12.5 mL of food eluate by centrifugation at 1,575 $\times$ g until the final volume reached 500 – 1000 µL. For an inoculum level of $10^4$ RT-PCR units of virus per 25 g of food, the recovery rates of the virus from lettuce, ham, and raspberries were approximately 20%, 5%, and 2%, respectively. Lee et al. (2012) used an ultrafiltration protocol to concentrate HAV from vegetable eluates. The Vivaspin 20 filter (100,000 norminal molecular weight limit; Sartorius stedium, France) was used in the study. Vegetable wash-off containing HAV in a 50-mL Vivaspin 20 tube was concentrated to a volume of 250 – 500 µL by centrifugation at a maximum speed of 10,000 $\times$ g at 4°C for 10 to 40 min, depending on the viscosity of the sample. The extracted viral RNA was tested by real-time RT-PCR. For an inoculum level of $10^4$ copies/mL of HAV, the ultrafiltration method demonstrated a recovery of 60% and 40.8%
of the virus in wash-off of 5 g of lettuce and perilla leaves, respectively. Liu et al. (2012) built an ultrafiltration procedure for simultaneous recovery of MS2 and φX174 bacteriophages, *C. perfringens* spores, *E. coli*, and *Cryptosporidium parvum* oocysts from 10 L of reclaimed water samples using two hollow fiber ultrafilters, Fresenius F200NR (Fresenius Medical care, Lexington, MA, USA) made of polysulfone and Baxter Exeltra Plus 210 (Baxter Healthcare Corp, Deerfield, IL, USA) made of cellulose triacetate. Both ultrafilters resulted in a recovery rate of ≥ 50% of seeded viruses, bacteria, and parasites.

### 1.4.2.4. Charged membrane filtration

Positively or negatively charged filters have been used to concentrate viruses from wash-off of contaminated foods. The principle of the method includes two steps: adsorption of viruses in food eluates on charged filters followed by elution of viruses off the filters into a small volume of suspension. Queiroz et al. (2001) used a two-step protocol to concentrate rotavirus from raw sewage and polluted creek water samples. The water samples were first subjected to filtration through positively charged Zeta Plus 60S filters (AMF Cuno Division, Meriden, CT, USA). The filter-bound viruses were eluted with 100 mL of a 3% beef extract-0.05 M glycine solution (pH 9.0) and then extracted with Vertrel XF (Du Pont) to remove PCR inhibitors. In this way, 8 out of 31 (25.8%) samples were tested positive for the virus. Brassard et al. (2005) also used the Zeta Plus 60S filter (Peacock, LaSalle, QC, Canada) to recover HAV and rotavirus in bottled spring water. The filter-bound viruses were eluted with a tryptose phosphate broth-glycine buffer (TPBG buffer, pH 9.0) and the eluates were further concentrated with Microsep® 100 (Pall Life Science, East Hills, NY, USA) to 150 µL. The RNAs were extracted using the RNeasy kit (Qiagen, Mississauga, ON, Canada). The detection
limits of RT-PCR for HAV and rotavirus were 0.1 TCID$_{50}$/mL and 0.001 TCID$_{50}$/mL, respectively. In another study conducted by the same research group, the Zeta Plus 60S filter was employed to absorb rotavirus, HAV, *E. coli*, *L. monocytogenes*, *C. jejuni*, and *S. Typhimurium* from spinach eluate and water. The microorganisms on spinach (25 g) were eluted by 225 mL of washing buffer (glycine 0.05 M, NaCl 0.14 M, pH 7.5) in a gentle shaking mode. The microbes in the spinach eluate and water were subjected to filtration, and the filter-bound microbes were eluted using 10 mL of TPBG buffer (pH 9.0). The viruses in the TPBG buffer were further concentrated using an Amicon centrifugal unit by centrifugation at 5,000 $\times$ g for 10 min before RNA extraction. This filtration technique allowed a detection level of $10^2$ CFU/g for *S. Typhimurium*, *E. coli*, *L. monocytogenes* and *C. jejuni*, and $10^1$ PFU/g for HAV and rotavirus (Brassard et al., 2011). Kovač et al. (2009) used a positively charged convective interaction media® quaternary amine (CIM-QA) monolithic column (BIA Separations, d.o.o., Ljubljana, Slovenia) to concentrate HAV from 1.5 L of bottled water. The bound viruses were eluted from the monolith using 1 M NaCl to a final volume of 15 mL. The elution was concentrated further using an ultracentrifugation at 85,750 $\times$ g at 4 °C for 1 h. The pellet was subjected to RNA extraction using QIAamp Viral RNA Mini Kit (Qiagen, Hilden, Germany). The combined concentration method resulted in recovery rates of HAV between 30% and 40% depending on the loading level of the virus and a detection limit of 10 TCID$_{50}$ of HAV/1.5 L of water. Coudray et al. (2013) used a Zetapore 47 mm positive-charged membrane (Cuno Filtration SAS 3M, Cergy Pontoise, France) of pore size 0.45 µm to concentrate HAV in eluates (40 mL) from lettuce (25 g). The filtration was performed under vacuum and the flow rate used during filtration was approximately 40 mL/6 – 7 min. Then, the filters were placed in a 60 mm diameter Petri dish and incubated with 3 mL of lysis buffer.
from the NucliSens® easyMAG™ kit (BioMérieux, Marcy l’Etoile, France) used for RNA extraction. The RNA was tested by real-time RT-PCR and the detection limit of HAV was 1 PFU/25 g of lettuce. The recovery rates of HAV were between 0.88% and 5.58% depending on the contamination level of the virus in each sample. In a study by Morales-Rayas et al. (2009), a positively charged Disruptor filter (Ahlstrom Filtration, L.L.C., Mount Holly Springs, PA, USA) was used to recover MNV-1 from the eluates from raspberries, strawberries, lettuce, green onions, and mussels. Fifty mL of an elution buffer, 0.1 M Tris-HCl (pH 7.0)-1 M NaCl, was used to detach viral particles from food surface and the elution buffer was subjected to the Disruptor filter. The absorbed viral particles were eluted from the filter using 500 µL of glycine buffer (3% beef extract and 0.37% glycine, pH 9.0) and tested by real-time RT-PCR. The detection limits of MNV-1 were 10 PFU/50 g of fresh produce and 10^5 PFU/10 g of mussel, respectively. The same protocol was also used to simultaneously recover HAV and NoVs in fresh produce. The RNAs were tested using a multiplex real-time RT-PCR and the detection limit of each virus was 10^2 PFU/50 g of produce (Morales-Rayas et al., 2010).

Katayama et al. (2002) used an HA negatively charged membrane filter (Nihon Millipore) with a 0.45 µm pore size and 47 mm diameter to concentrate poliovirus from 1 L of seawater. The seawater was passed through the membrane at a flow rate of 100 mL/min. The membrane was then rinsed with 200 mL of 0.5 mM H_2SO_4 (pH 3.0) followed by elution of bound viral particles with 5 mL of 1 mM NaOH (pH 10.5 – 10.8). The neutralized eluate was further concentrated to a final volume of 1 mL with a Centriprep Concentrator 50 System (Nihon Millipore). Tested by plaque assay, 61% – 73% of the poliovirus could be recovered from the
experimentally contaminated seawater. Villar et al. (2006) reported that the method used by Katayama et al. (2002) was suitable for concentrating HAV in mineral water but not in tap water. For concentrating HAV in 2500 mL of tap water, 1200 mg/L of MgCl$_2$$\cdot$6H$_2$O was added to the tap water and pH of the water was adjusted to 5.0, followed by the filtration through a negatively charged Stericup$^\circledR$ filter (Millipore, Bedford, MA, USA) with a pore size of 0.45 µm. The bound viral particles were eluted with urea-arginine phosphate buffer (pH 9.0). The eluates were further concentrated using a flocculation method with 1 M MgCl$_2$$\cdot$6H$_2$O. After centrifugation at 3,000 × g for 30 min, the pellets were suspended in a final volume of 800 µL of distilled sterile water. The genome copies of HAV tested by real-time RT-PCR in the concentrated sample were about twice as many as in the original sample. Fumian et al. (2009) used a protocol to concentrate NoVs from cheese (15 g) and lettuce (25 g). The viral particles were eluted from food surface using PBS and then absorbed onto a negatively charged Stericup$^\circledR$ filter (Nihon Millipore, USA). The following steps were the same as described by Katayama et al. (2002). The recovery rates of NoVs ranged from 5.2% to 72.3% in lettuce and from 6% to 56.3% in cheese. Corrêa & Miagostovich (2013) used the similar protocol to Fumian et al. (2009) to concentrate NoVs from lettuce (25 g). In their protocol, 250 mL of glycine buffer (pH 9.5) was used to elute the virus from the surface of lettuce and concentrated to a final volume of 2 mL. For an inoculum level of $10^6$ genomic copies/25 g of lettuce, 18.84% of NoVs could be recovered. De Keuckelaere et al. (2013) reported the application of a negative-charged membrane filtration method for the concentration of MNV-1, HAV, NoVs, and rotavirus from 1 – 5 L of various types of irrigation water. Briefly, pH of the water was adjusted to 3.5 with 1 M HCl, and MgCl$_2$ was added to a final concentration of 0.05 M, and then the water was filtered through glass fiber
filters to delay clogging of negative-charged filters. A HAWP14250 negatively charged membrane (Millipore, Ireland) with a 0.45 µm pore size and a 142 mm diameter was used in a pressure pump system for virus adsorption. After filtration, the membranes were incubated with 70 mL of an elution buffer (0.05 M KH$_2$PO$_4$, 1.0 M NaCl, 0.1% (v/v) Triton X-100, pH 9.2) for 10 min and then the viruses were eluted from the membranes by applying pressure. The pH of the elution was neutralized and then the viruses were concentrated again using a PEG precipitation method. The RNAs extracted using the NucliSens® EasyMAG™ system (Biomérieux, Boxtel, the Netherlands) were tested by real-time RT-PCR. For an inoculum level of about 6 – 7 genome copies/L of water, the recovery rates of MNV-1, HAV, NoV (genogroup I), NoV (genogroup II), and rotavirus were 4.76% – 21.87%, 14.49% – 31.11%, 4.75% – 23.90%, 10.67% – 27.51%, 0.03% – 9.81%, respectively, depending on water types.

1.4.2.5. Magnetic bead separation

Paramagnetic beads coated with anti-virus antibodies were used to capture viruses in food or water samples. Monceyron & Grinde (1994) used a kind of polystyrene magnetic bead, Dynabeads M-280 (2.8 µm diameter, Dynal, Oslo, Norway), coated with monoclonal antibodies (K3-2F2) against surface epitopes of HAV, to concentrate HAV from 1 mL of polluted river water and seawater. The beads were incubated with the samples for 2 h at 37°C and trapped on the side of the tube using a high power magnet (Dynal, Oslo, Norway) to remove the supernatant. The beads were resuspended in 1 – 3 µL of PCR buffer and RNA was released by heating at 99°C for 5 min and tested by RT-PCR. The detection limits of HAV in river water and seawater were 10 TCID$_{50}$/mL and 100 TCID$_{50}$/mL, respectively. Casas & Suñén (2002) used paramagnetic beads with covalently linked goat antihuman
Immunoglobulin G (IgG) to capture poliovirus type 1 and HAV in 1 L of sewage. The sample was concentrated to 16 mL using a PEG precipitation method firstly and heated at 99°C for 2 min to destroy the natural contaminated viral particles. The viruses were seeded into the concentrate. Then, 500 µL of the contaminated concentrate were incubated with IgG-coated beads for 2 h with gentle mixing. The virus-beads complex was washed three times with PBS-0.05% Tween 80 and finally was concentrated to the bottom of tube by centrifugation at 7,000 × g for 2 min. The complex was resuspended in 50 µL of PCR buffer and viral RNA was released by heating at 95°C for 5 min. The beads were removed by centrifugation at 13,000 × g for 2 min and the supernatant was analyzed immediately by RT-PCR. The detection limits of poliovirus and HAV were 0.1 – 1 PFU and 1 most probable number of cytopathogenic unit (MPNCU) per 500 µL of concentrated sample, respectively. In another study by the same research group, the IgG-coated paramagnetic beads were used to concentrate HAV from clam concentrates obtained from 25 g of contaminated clams by PEG precipitation or ultracentrifugation method. The procedure for IgG-coated bead capture was the same as Casas & Suñén (2002). The detection limits of HAV for the two methods were the same to be 0.1 MPNCU, but up to 8 g of clam tissue could be processed by the PEG precipitation plus immunomagnetic capture method, whereas up to 4 g of clam tissue could be processed by the ultracentrifugation plus immunomagnetic capture method. However, the former protocol involved numerous steps and was time-consuming. Abd El Galil et al. (2004) used an immunomagnetic separation method to recover HAV from ground water concentrate. In the study, 1,000 L of ground water were filtered through an electropositive MDS filter (AMF CUNO, Meriden, Conn.) and the filter cartridge was flushed with 1 L of 1.5% (w/v) beef extract V containing 0.05 M glycine (pH 7.5). The eluate was concentrated by adjusting the
pH to 3.5 and then centrifugation at $4,000 \times g$ for 15 min. The resulting pellet was resuspended in 20 mL of PBS and HAV was inoculated into the concentrates. Magnetic beads coated with a human polyclonal HAV antibody were added to the concentrates to recover HAV by incubation at room temperature for 2 h. The beads were recovered with a strong magnetic particle separator stand. Viral RNA was released by heating at 99°C for 5 min in 13 μL of RT-PCR buffer and subjected to RT-PCR assay. The detection limit of HAV was 20 PFU in the ground water concentrates. Shan et al. (2005) used Dynabeads (Dynal, Great Neck, New York) coated with monoclonal antibodies against HAV (anti-HAV 1009; Argene, New York) to recover HAV from 1 mL of green onion and strawberry rinses. The beads were incubated with the produce rinses for 1.5 h, collected and resuspended in 50 μL of PBS, and then boiled for 5 min to lyse viral capsid. The beads were harvested by centrifugation at $12,000 \times g$ for 1 min and the supernatant was analyzed using real-time PCR. In both green onion and strawberry samples, the sensitivity of the assay was 10 PFU/mL. Two different Dynabeads, M-280 (2.8 μm diameter) and Dynabeads MyOne (1.05 μm diameter), were compared but no significant difference in efficiency was found in recovering HAV. Papafragkou et al. (2008) reported a method of using cationically charged magnetic particles with a commercially available automated capture system (Pathatrix™, Matrix Microscience) to concentrate HAV from 25 g samples of artificially contaminated lettuce, strawberries, green onions, deli-turkey, oysters, and cake with frosting. For deli-turkey, oysters, and cake with frosting, because of the complexity of sample matrices, these samples were pre-processed before magnetic capture. Twenty-five g of virus-inoculated samples were soaked in 225 – 245 mL of glycine–saline buffer (0.05 M glycine, 0.14 M NaCl, pH 9.0) in a sterile Whirl-pak filter bag and stomached at 260 revolutions per minute (rpm) for 2 min. The filtrate was
centrifuged to remove food debris and the viral particles in supernatant were precipitated using 8% – 10% PEG 6,000 and 0.5 M NaCl. The PEG precipitates were resuspended in 240 mL of glycine-saline buffer (pH 7.0) prior to magnetic capture. Each produce eluate or treated sample of deli-turkey, oysters, and cake with frosting was added with 50 µL of magnetic beads and circulated in the Pathatrix™ system for 30 min. Viral RNA was extracted from the captured beads and subjected to RT-PCR and dot-blot analysis. Detection limits varied according to the product but in most cases, the virus could be consistently detected at input levels corresponding to 10² PFU/25 g of food sample. For lettuce and green onion, detection was possible at levels as low as 10⁻² PFU/25 g. The Pathatrix™ system was also used by Hirneisen, et al. (2009) for the concentration of HAV, Aichi virus, feline calicivirus (FCV) and raccoon poxvirus from artificially contaminated salsa and milk (1% fat). A 25-g sample of salsa seeded with viruses of a titer of 10⁷ – 10⁸ TCID₅₀/g was added to 225 mL of distilled water in a sterile stomacher bag. Milk (250 mL) was inoculated with viruses of a titer of 10⁷ – 10⁸ TCID₅₀/mL in a sterile stomacher bag. Samples were run on the Pathatrix™ system for 60 min at room temperature (25°C) using 50 µL of positively charged cationic beads (ZCCB-CAT, Matrix MicroScience). After recirculation, the beads were washed with sterile water and recovered on a magnetic rack. Infectivity was determined by a TCID₅₀ assay. It was found that HAV and Aichi virus could be recovered efficiently by the system but FCV and raccoon poxvirus could not be recovered. In this study, Aichi virus recovery was between 31% and 62%, and HAV recovery was 36% – 70% in all trials. The detection limit of HAV and Aichi virus in milk was 10⁶ TCID₅₀/mL and 10³ TCID₅₀/mL, respectively.

Researchers have compared the efficiency of the above-mentioned methods to concentrate viruses from different types of foods and found a single method could not always demonstrate...
the best efficiency for all foods. Summa et al. (2012) compared the efficiency of four virus recovery methods (ultrafiltration, immunomagnetic separation, ultracentrifugation, and PEG precipitation) to concentrate NoV in the eluates from fresh lettuce, sliced ham, and frozen raspberries. For an inoculum level of $10^4$ RT-PCR units of virus per 25 g of food, the recovery rates of the virus given by the ultrafiltration method from lettuce, ham, and raspberries were approximately 20%, 5%, and 2%, respectively. The ultracentrifugation method yielded the highest recovery efficiencies in ham (70%), whereas the PEG precipitation method recovered the highest yield of NoV from raspberries (29%). The immunomagnetic separation method was applied using a commercial NoroCheck IMS kit (Kim Laboratories, USA) and yielded less than 5% recovery of the virus in all food types. Lee et al. (2012) compared the efficiency of three different methods, PEG precipitation, ultrafiltration, and immunomagnetic separation, to concentrate HAV from vegetable eluates. The ultrafiltration method demonstrated the highest efficiency giving a recovery of 60% and 40.8% of HAV in wash-off of lettuce and perilla leaves, respectively. Whereas, the PEG precipitation method showed an average recovery rate of 5.65% of HAV in vegetable wash-off and the immunomagnetic separation method only recovered 0.65% of the virus. Coudray et al. (2013) compared PEG precipitation and electro-positive charged membrane filtration method in concentrating HAV from lettuce eluate, and found that both methods demonstrated an identical limit of detection, although the filtration method required less processing time. Di Pasquale et al. (2010) compared ultracentrifugation and ultrafiltration method in concentrating HAV from 1.5 L of bottled water and found that ultracentrifugation-based method resulted in higher recovery rate of HAV. Kovač et al. (2009) also reported that, in comparison with ultrafiltration-based method, ultracentrifugation-based method improved the recovery of HAV by approximately 20% from
water. Suñén et al. (2004) compared the efficiency of a PEG precipitation method and an ultracentrifugation method to concentrate HAV from clams, and found that the PEG precipitation method was more effective in removing inhibitors but it was time consuming. In some research, two different concentration methods were even combined to improve the overall recovery efficiency of virus (Kovač et al., 2009; Villar et al., 2007).

Each of the aforementioned methods has their own disadvantages. For ultracentrifugation, specialized apparatus needs to be used, and the high speed makes this method dangerous, requiring specialized personnel (Stals et al., 2012). Filtration-based methods are frequently used to process contaminated water or the rinse solution with less food residue (Brassard et al., 2005; De Keuckelaere et al., 2013; Di Pasquale et al., 2010; Queiroz et al., 2001; Villar et al., 2006). As to the charged-membrane filtration method, it was reported that a considerable number of viral particles passed through the pores of membranes instead of being trapped by the electrostatic force (Di Pasquale et al., 2010). PEG precipitation is time-consuming, requiring overnight precipitation at 4°C to give ideal results (Jones & Johns, 2009). As described previously, the immumagnetic capture method is used for recovering viruses from samples with small volumes of ≤ 1 mL. The method is not suitable for routine use in processing samples with large volume because of the high cost of antibodies, and it cannot guarantee the capture of all HAV strains due to the regular emergence of new antigenic variants (Chironna et al., 2004; Costa-Mattioli et al., 2002a; Gharbi-Khelifi et al., 2007; Pérez-Sautu et al., 2011b; Sánchez et al., 2002). Therefore, there is a need to improve the existing approaches or develop novel methods that are more cost-effective, easier to perform, or more rapid to concentrate foodborne viruses.
1.4.3. Magnetic nanoparticle capture method

Recently, iron oxide (Fe$_3$O$_4$) magnetic nanoparticles (MNPs) have attracted the attention of scientists and have rapidly gained importance in biotechnology. In comparison with microscale materials, nanoscale materials have some special properties such as higher surface-to-volume ratio, rapid diffusion, good dispersability, and many unique size-dependent qualities (Yang et al., 2008).

Numerous different surface-modified Fe$_3$O$_4$ MNPs have been applied in various fields. In clinical applications, Fe$_3$O$_4$ MNPs have demonstrated great potentiality in drug delivery, gene transfer, and magnetic resonance imaging (MRI) (Colombo et al., 2012). The Fe$_3$O$_4$ MNPs also showed good efficiency in separation of chemicals such as heavy metals and dyes in environmental samples. In a study, diethylenetriamine functionalized Fe$_3$O$_4$ MNPs (11.2 nm diameter) were used to remove heavy metal ions, such as Cu(II) and Cr(VI), from water. The concentrated metal ions were measured using an Atomic Absorption Spectrometer. The adsorption capacity of Fe$_3$O$_4$ MNPs for Cu(II) and Cr(VI) was 12.43 mg/g and 11.24 mg/g, respectively (Huang & Chen, 2009). A superparamagnetic nanocomposite consisting of Fe$_3$O$_4$ MNPs (10 nm diameter) and sodium alginate supported tetrasodium thiocalix[4]arene tetrasulfonate (Fe$_3$O$_4$@TSTC[4]AS-s-SA) was used to remove heavy metal ions, such as Cu(II), Cd(II), Pb(II), Co(II), Ni(II) and Cr(III) ions, from wastewater followed with detection using an Atomic Absorption Spectrometer. The nanocomposite demonstrated good efficiency in removing heavy metals in wastewater and the order of sorption efficiency for the heavy metal ions was: Pb(II) > Cd(II) > Cu(II) > Cr(III) > Co(II) > Ni(II) (Lakouraj et al., 2014). Chen et al. (2014) fabricated chitosan/polyacrylic acid multilayer onto Fe$_3$O$_4$ MNPs (200 –
and used the surface-modified MNPs to remove methylene blue and crystal violet in wastewater. The absorbed dyes were measured by a UV-vis spectrophotometer. The adsorption capacity of the MNPs was found to be 305.8 mg/g and 243.6 mg/g for methylene blue and crystal violet, respectively. Mohammadi et al. (2014) used sodium alginate-coated Fe$_3$O$_4$ MNPs (12 nm diameter) to remove malachite green from aqueous solutions and found that the adsorption capacity of the MNPs for the dye was 47.84 mg/g.

The Fe$_3$O$_4$ MNPs have been found with good potentials in rapid capture of bacterial pathogens. El-Boubbou et al. (2007) used mannose-coated Fe$_3$O$_4$ MNPs (about 10 nm diameter) to capture *E*. *coli* ($10^3 - 10^7$ cells/mL) in PBS. The captured bacterial cells were detected using fluorescence microscope. It was found that 65% of *E*. *coli* cells could be captured even with an incubation time of the MNPs and bacterium for 5 min. The Fe$_3$O$_4$ MNPs have also shown high efficiency in isolating pathogenic bacteria from foods. Ravindranath et al. (2009) used antibody-coated Fe$_3$O$_4$ MNPs (17 to 50 nm diameter) to isolate *E*. *coli* O157:H7 and *S*. Typhimurium from milk and spinach, followed by detection using a portable mid-infrared (mid-IR) spectrophotometer. These researchers were able to detect both pathogens specifically in 30 min with a detection limit of $10^4 - 10^5$ CFU/mL. Cheng et al. (2009) reported a rapid, specific, and sensitive method of separating *E*. *coli* from pasteurized milk using Fe$_3$O$_4$ MNPs (about 20 nm diameter) functionalized with specific anti-*E*. *coli* antibody. The isolated *E*. *coli* was detected by adenosine triphosphate (ATP) bioluminescence within 1 h with a detection limit of 20 CFU/mL. Lee et al. (2014) incorporated Fe particles (40 nm diameter) inside the silica mesopores and then coated Ni$^+$ on the surface of the mesopores. This Ni$^+$-heterogeneous magnetic mesoporous silica (Ni-
HMMS) could bind with nickel-binding protein on the cell membrane of some pathogenic bacteria, for example, *E. coli* O157:H7. In this study, the Ni-HMMS was used to isolate *E. coli* O157:H7 in milk. After incubation and magnetic separation, the collected Ni-HMMS was re-suspended in 10 mM of Tris-hydrochloride buffer containing 1.0 mM EDTA (TE buffer, pH 7.5) and the bound cells were lysed through heating at 95°C for 5 min. The clear cell lysates were collected from Ni-HMMS surface by magnetic attraction and tested directly by RT-PCR. The Ni-HMMS was successful in separating *E. coli* O157:H7 in milk and 10 CFU/mL of the cells could be detected.

The Fe$_3$O$_4$ MNPs were also used to capture genomic DNA of lysed bacterial cells isolated from foods. Amagliani et al. (2006) immobilized an oligonucleotide sequence specific to the *hlyA* gene of *L. monocytogenes* to the surface of MNPs (30 – 100 nm diameter), and used the modified nanoparticles to capture the DNA of *L. monocytogenes* isolated from milk prior to PCR detection. The detection sensitivity of DNA captured by the MNPs was one log higher than that obtained using Dynabeads M-280 and M-270 (both 2.8 µm diameter). Bai et al. (2013) used amino-modified silica-coated Fe$_3$O$_4$ MNPs (14 nm diameter) to capture genomic DNA of *S. enteritis* and *L. monocytogenes* collected from 10 mL of milk. The bacterial cells were collected by centrifugation at 6,000 × g for 20 min and resuspended in 500 µL of 10 mM TE buffer (pH 9.0) with 5% (w/v) Triton X-100. The genomic DNA was released from bacterial cells by heating at 100°C for 10 min, captured by the MNPs through incubation at 60°C for 30 min, and then was detected by PCR. The results showed that 8 CFU/mL of *S. enteritis* and 13 CFU/mL of *L. monocytogenes* could be detected.
In comparison with magnetic microbeads (MMBs), MNPs showed improvement in isolating bacteria from foods. Yang et al. (2007) used Fe₃O₄ MNPs (50 nm diameter) coated with rabbit anti-\textit{L. monocytogenes} to concentrate \textit{L. monocytogenes} from milk in combination with plate counting and real-time PCR detection. The researchers compared the capture efficiency of immunomagnetic nanoparticles and Dynabeads (around 2.8 µm diameter) coated with the same antibody to isolate \textit{L. monocytogenes} from milk, and found that the capture efficiency of nanoparticles was 1.4 to 26 times higher than that of Dynabeads depending on the amount of \textit{L. monocytogenes} inoculated into milk. Varshney et al. (2005) compared the efficiency of MNPs (145 nm diameter) coated with polyclonal goat anti-\textit{E. coli} antibodies with that of MMBs (2.8 µm diameter) to isolate \textit{E. coli} O157:H7 from ground beef (25 g). Capture efficiencies of 98%, 98%, and 97% for \textit{E. coli} O157:H7 cells at inoculum levels of \(3.6 \times 10^3\), \(4.2 \times 10^5\), \(3.6 \times 10^7\) CFU/0.5 mL, respectively, were achieved with the MNPs while the corresponding numbers for MMBs were 92%, 90%, and 83%, respectively. As for the detection of \(3.6 \times 10^5\) CFU/0.5 mL of \textit{E. coli} O157:H7, MNPs were able to capture 96.3% of cells within 15 min, whereas MMBs needed more than 1 h to achieve the same capture efficiency.

In addition to separation and concentration of pathogens from food matrices, the MNPs were also employed to detect the concentrated pathogens. Pal & Alocilja (2009) activated the surface of Fe₂O₃ MNPs (20 nm diameter) with electrically active polyaniline and then conjugated mouse monoclonal anti-\textit{Bacillus anthracis} antibody to the surface of Fe₂O₃ MNPs. The modified MNPs were used to capture \textit{B. anthracis} spores in romaine lettuce, ground beef, and ultra-pasteurized whole milk samples. The captured bacterial spores on the conductive
MNPs were detected using a biosensor detection system by measuring the decrease of electrical resistance across the silver electrodes caused by the MNPs. It was shown that concentrations as low as $4.2 \times 10^2$ spores/mL were detectable. Kanayeva et al. (2012) developed an MNP-based method for rapid separation and detection of \textit{L. monocytogenes} in food samples. In the study, polyclonal rabbit anti-\textit{L. monocytogenes} antibodies were conjugated to Fe$_3$O$_4$ MNPs (30 nm diameter) and the antibody-coated MNPs were used to recover \textit{L. monocytogenes} in 1 mL of wash solution from contaminated lettuce and ground beef, or in 1 mL of milk. The antibody-coated MNPs were incubated with food samples for 2 h, collected by a magnetic separator, and then resuspended in 50 µL of PBS. Thirty µL of the separated samples were injected into a microfluid chip designed with an interdigitated microelectrode-based impedance immunosensor in the presence of 0.1 M mannitol solution. The change of the magnitude of impedance for the sample containing the MNPs-\textit{L. monocytogenes} complex was measured. The results showed that, without pre-enrichment, $10^4$ CFU/mL of \textit{L. monocytogenes} in lettuce, milk, and ground beef samples could be detected within 3 h. Zhao et al. (2013) used anti-\textit{Cronobacter sakazakii} antibody-coated Fe$_3$O$_4$ MNPs (60 nm diameter) to collect \textit{C. sakazakii} in infant milk powder and cheese. The antibody-modified MNPs were incubated with the samples for 2 h and then 1.5 mL of the sample was subjected to nuclear magnetic resonance (NMR) analysis for the detection of the captured bacteria. The detection limits of the \textit{C. sakazakii} in milk powder and cheese were 1.1 and 11 MPN, respectively. Sung et al. (2013) developed a method of using antibody/gold nanoparticle/Fe$_3$O$_4$ MNPs (100 nm diameter) to isolate \textit{S. aureus} in milk and detect the bacterial cells using a colorimetric method. In their study, gold nanoparticles (AuNPs) were conjugated with Fe$_3$O$_4$ MNPs firstly, and then anti-\textit{S. aureus} antibodies were coupled to the
gold nanoparticles. Twenty µL of the antibody/AuNPs/Fe₃O₄ MNPs nanocomposites were mixed with 10 µL of artificially contaminated milk sample and 70 µL of PBS for 30 min. The nanocomposites were collected with a magnet and resuspended in 100 µL of PBS. Fifty µL of the processed sample was filtered through a membrane with 0.8 µm diameter pores. The cell-nanocomposite complex remained on the surface of the membrane, while the unbound nanocomposites passed through the membrane. After the gold enhancement, the cell-nanocomposite rendered a visible color. The assay required only 40 min to get the results. The detection limit of *S. aureus* was $1.5 \times 10^5$ CFU in milk sample. A similar assay was used for the immunoseparation of *S. Typhimurium* and colorimetric detection of the magnetic captured cells in lettuce and cabbage. The assay demonstrated a detection limit of 100 cells/g of vegetables (Shim et al., 2014).

Although the Fe₃O₄ MNPs have been used to capture foodborne bacteria with higher efficiency than MMBs, there are no reports about the applications of the MNPs in capturing viruses in foods. Therefore, we planned to use Fe₃O₄ MNPs (20 – 30 nm diameter) in this research to capture viruses from food samples. HAV was used as a model virus to build the method. Because of the high price of antibody, cationic Fe₃O₄ MNPs were used in the current study for the concentration of HAV. Protamine is a unique food protein found in fish sperm. Because of the extremely high composition of L-arginine, a basic amino acid, this protein is highly positively charged (Hoffmann et al., 1990). It is an important medical product and used to neutralize heparin following certain surgical procedures and in the treatment of heparin overdose (Hoffmann et al., 1990). The isoelectric point (pI) of protamine was reported to be 12.1 – 13.8 (Hoffmann et al., 1990; Tang et al., 1993). The pI of HAV was reported to be 2.8
(Michen & Graule, 2010). Thus, when the pH is between 2.8 and 12.1, HAV and protamine is negatively and positively charged, respectively, resulting in the formation of a complex by electrostatic attractive force. Based on this principle, protamine-coated Fe₃O₄ MNPs were investigated in this study for capturing HAV from food.

1.4.4. PEG dialysis method

It has been shown that water can be removed from casein dispersions in a dialysis tube immersed in a reservoir of PEG, resulting in the casein dispersion being concentrated (Bonnet-Gonnet et al., 1994; Bouchoux et al., 2009; Parsegian et al., 1986). The MWCO of the dialysis tube is selected to allow water, ions, and small organic molecules other than casein and PEG to pass through the tube (Bouchoux et al., 2009). Therefore, dialysis of HAV suspensions (40 – 50 mL) against PEG solutions was used in the current research as a method for the concentration of viruses eluted from foods. The molecular weight of HAV is 5,298 KDa (Martin & Lemon, 2006; Ross & Anderson, 1991). In this study, to ensure no permeation of the viral particles across the dialysis tube, an MWCO of 3.5 KDa was employed, and the PEG had an average molecular weight of 20 KDa. Compared with the currently used virus concentration methods, this method is much cheaper and simpler. Basically, this method has the potential to be used anywhere, as it does not require any sophisticated infrastructure.

1.5. Methods of foodborne virus detection

Virus detection is based on two principles: the detection of infectious viruses by propagation in cell culture; or the detection of viral genomes by molecular biological techniques.
1.5.1. Cell culture

The virus cell culture method is based on the formation of cytopathic effects (CPE), followed by the counting of viruses by plaque assay, the MPN or TCID\textsubscript{50} (Hamza et al., 2011). Since the 1940s, numerous attempts have been made to propagate HAV in primary cell cultures and continuous cell lines. However, most of these efforts have been unsuccessful because HAV does not normally cause CPE. Later, the serial passage of wild-type virus strain resulted in the generation of cell culture adapted strains that typically replicate faster than the parental wild-type strain, but remained non-cytopathic. The further serial passage of cell culture-adapted non-cytopathic strain HM175 resulted in the emergence of faster growing strains, HM175/24A and HM175/18f, capable of inducing CPE during virus replication in cell lines such as the cloned line of fetal rhesus monkey kidney cells (FRhK-4), African green monkey kidney cells (AGMK) or the continuous cell line of AGMK, B-SC-1 (Cromeans et al., 1987; Daemer et al., 1981; Yi & Lemon, 2002). These two CPE-inducing strains have been widely used to experimentally contaminate foods for building the methods for HAV detection. However, cell culture based methods for these two strains are complicated and tedious and the emergence of CPE takes at least 8 days (Richards & Watson, 2001). For the aforementioned reasons, currently, the cell culture methods are still not suitable for rapid detection of HAV in food.

1.5.2. Immunoassay

In 1969, the development of immune electron microscopy (IEM) was reported for visualization of the interaction between virus and antibody (Almeida & Waterson, 1969). In the 1970s, virologists started to apply the IEM technique for the detection of HAV (Cook et al., 1976; Feinstone et al., 1973; Gravelle et al., 1975; Hollinger et al., 1975). Subsequently,
different categories of immunoassays with greater simplicity and sensitivity were developed, such as radioimmunofocus assay (RIFA) (Anderson, 1987; Lemon et al., 1983), enzyme-linked immunosorbent assay (ELISA) (Nasser & Metcalf, 1987), luminescent immunoassay (LIFA) (Richards & Watson, 2001), immunofluorescent confocal microscopy (Kukavica-Ibrulj et al., 2003), and infrared fluorescent immunofocus assay (IR-FIFA) (Counihan et al., 2006). These methods are still time-consuming and labor-intensive, and the sensitivity of these assays was reported to between $2 \times 10^5$ and $10^6$ viral particles/mL (Kukavica-Ibrulj et al., 2003; Nasser & Metcalf, 1987). As stated previously in Section 1.4, the contamination level of HAV in most food is below the detection limit of these immunoassays.

1.5.3. Molecular biological methods

1.5.3.1. PCR technique

PCR technique was discovered by Kary Mullis in 1983 (Mullis et al., 1986). In this technique, the reaction cycles consist of three steps: (1) denaturation of the dsDNA; (2) annealing of short DNA fragments (primers) to ssDNA; and (3) extension of the primers with a thermostable DNA-polymerase (Mullis et al., 1986). As long as there is an excess of primers, nucleotides and enzyme, the initial DNA can be copied exponentially. At the end of the amplification, the product can be run on a gel for detection or identified by southern blotting (Scheu et al., 1998). Since the early 1990s, this technique has been used for the detection of various pathogens in foods (Scheu et al., 1998). The first report of the application of PCR to detect HAV in oysters was in 1993 (Atmar et al., 1993). The specificity of the assay is determined by the sequence of the primers that are unique for the target microorganism (Scheu
et al., 1998). The sensitivity of the assay is not only determined by the reaction conditions, but also to a great extent affected by the food matrix (Scheu et al., 1998). Some food components, such as Ca\(^{2+}\) (Bickley et al., 1996), NaCl, KCl, phenol (Davalieva & Efremov, 2010), polysaccharides (Monteiro et al., 1997), collagen (Kim et al., 2000), proteinases (Bickley et al., 1996), and glycogen (Atmar et al., 1993), can inhibit the sensitivity of PCR. These inhibitors can prevent amplification by direct interaction with DNA (Opel et al., 2009). They can also interfere with DNA polymerases, blocking the enzyme activity (Opel et al., 2009) or they interact with Mg\(^{2+}\), which is a critical cofactor of DNA polymerase, and reduce the availability of the Mg\(^{2+}\) to DNA polymerase (Yang et al., 2004). The inhibitors also interfere with the activity of reverse transcriptase for cDNA synthesis (Hata et al., 2011). The approach to avoid PCR inhibition is separating target microorganisms from the inhibitors and purifying nucleic acids of the microorganisms. However, there are some inhibitors that are not possible to be eliminated, and other options need to be adopted to improve the sensitivity of the technique. These options are stated as follows.

Proper thermostable DNA polymerase that is less sensitive to inhibition could be used in the PCR amplification. It is reported that *Thermus aquaticus* (*Taq*) DNA polymerase is easy to be inhibited by numerous substances mentioned previously. However, the tolerance of *Thermus thermophiles* (*Tth*) and *Thermotoganeapolitana* (*Tne*) DNA polymerase to inhibitors is 10 to 30 times higher than that of *Taq* DNA polymerase (Davalieva & Efremov, 2010). *Thermus flavus* (*Tfl*) DNA polymerase was also found to be more tolerant to inhibitors than *Taq* DNA polymerase (Wiedbrauk et al., 1995). Using diluted RNA or DNA as PCR template might reduce inhibitory effect. Summa et al. (2012) found that a ten-fold dilution of NoV RNA
extracted from raspberries could clearly improve the sensitivity of PCR. Some enhancing agents, for example, bovine serum albumin (BSA) or T4 gene 32 protein, could be added to the reaction to increase the yield of PCR product. Plante et al. (2011) reported that isolation of viruses from spinach and precut lettuce had the potential to generate RNA extracts that were strongly inhibitory to real-time RT-PCR for the detection of NoV GII, HAV, HEV, and rotavirus. While, the addition of BSA to those reactions restored a positive signal in all cases. Chandler et al. (1998) found that the incorporation of T4 gene 32 protein into the reverse transcription phase of RT-PCR reaction increased the RT-PCR product yield by as mush as 483%. Monpoeho et al. (2000) reported that, when a real-time RT-PCR was used for the detection of poliovirus RNA in urban sludge samples, the detection limit of the virus was $1.6 \times 10^4$ copies/reaction, and with the addition of T4 gene 32 protein in the reaction, the detection limit of the virus was improved to be $1.6 \times 10^1$ copies/reaction. Additional nucleic acid amplification systems, such as nested PCR (Roux 1995) and real-time PCR, have been reported to be more sensitive than conventional PCR (Bart et al., 2007; Casas et al., 2007; Villar et al., 2007).

Real-time PCR is a technique that combines DNA amplification with the immediate detection of the products together. This technique was invented in the early 1990s (VanGuilder et al., 2008). It removes the significant contamination risk caused by opening tubes for post-PCR manipulation, is less time-consuming than gel-based analysis, and can give quantitative results. Because of its high speed, simplicity, and minimization of contamination, this technique has been widely used in the field of food virology in the last decade (Bosch et al., 2011). Several different fluorescent reporter technologies of real-time PCR have been developed for the
detection of viruses in food or environmental samples, such as SYBR Green I-based real-time PCR (Casas et al., 2007), probe-based assays including Taqman® real-time PCR (Costafreda et al., 2006; Houde et al., 2007; Jothikumar et al., 2005) and molecular beacon-based real-time PCR (Abd El Galil et al., 2004). Probe-based assays are more sensitive and rapid than SYBR Green I-based assay. The Taqman® real-time PCR has been widely applied for HAV detection, and several primer-probe sets have been validated (Costafreda et al., 2006; Costa-Mattioli et al., 2002b; Gardner et al., 2003; Houde et al., 2007; Jothikumar et al., 2005). As HAV is an RNA virus, the genome of HAV needs to be reverse transcribed to complementary DNA (cDNA) before a real-time PCR assay can be performed. This can be done in One-Step or Two-Step real-time RT-PCR format (Bianchi et al., 2011; Coudray-Meunier et al., 2013; Di Pasquale et al., 2010; Kovač et al., 2009; Uhrbrand et al., 2010). Even though real-time RT-PCR technology has made the detection of HAV easier and more rapid than conventional PCR, the problem of inhibition still exists (Bianchi et al., 2011; Dubois et al., 2007). Also, not all laboratories can afford an expensive thermal cycler used in real-time PCR. Thus, novel, robust, cost-effective, and sensitive methods are needed to solve the existing problems.

1.5.3.2. Loop-mediated isothermal amplification (LAMP) technology

LAMP technology was first described for the detection of nucleic acids by Notomi et al. (2000). The basic principle of the LAMP reaction is shown in Figure 1.3. This method employs Bst DNA polymerase, an enzyme derived from Geobacillus stearothermophilus (formally Bacillus stearothermophilus), with strand displacement activity (Nagamine et al., 2002). The amplification is conducted at a constant moderate temperature between 60 and 65°C and does not need an expensive thermal cycler. Aside from its isothermal character, LAMP has several important features: (1) LAMP shows exquisite specificity because of the involvement of four
or six primers: two inner primers (FIP and BIP), two outer primers (F3 and B3), and sometimes two loop primers (FLOOP and BLOOP). These primers recognize six or eight regions of the target (Notomi et al., 2000); (2) the product of LAMP consists of a mixture of stem-loop DNAs with various sizes, giving rise to distinct ladder-like banding patterns on an agarose gel (Notomi et al., 2000); (3) the LAMP reaction can be easily detected by visual endpoint observation of a white precipitate of magnesium pyrophosphate (Mori & Notomi, 2009), or of the color change of calcein (Tomita et al., 2008), SYBR Green I (Njiru et al., 2012; Tao et al., 2011), or hydroxynaphthol blue (Goto et al., 2009) involved in the reaction; (4) LAMP product can be monitored by a real-time measurement of the turbidity of magnesium pyrophosphate, a byproduct of DNA amplification (Mori et al., 2004) or the light output of ATP produced from inorganic pyrophosphate (PPi) (Gandelman et al., 2010), both of which are more cost-effective than a fluorescence-based assay; (5) the LAMP reaction is more tolerant to substances that typically inhibit PCR (Francois et al., 2011; Kaneko et al., 2007; Ou et al., 2012) and has demonstrated significantly higher sensitivity than real-time PCR for the detection of *Mycobacterium tuberculosis* in pleural fluid (Yang et al., 2011); and (6) portable devices, such as coin-size microfluidic chips, can be designed to fulfill the need for on-site detection using the LAMP technique in remote areas where resources are limited (Ahmad et al., 2011; Hsieh et al., 2012; Liu et al., 2011; Lucchi et al., 2010; Wu et al., 2011; Yi et al., 2014).
Figure 1.3. Schematic representation of the LAMP mechanism (Mori et al., 2004). (A) primer design for LAMP reaction; (B) starting structure producing steps of LAMP; (C) cycle amplification step.

The method combining reverse transcription, loop-mediated isothermal amplification (RT-LAMP) and bioluminescence assay in real-time (BART) is named RT-LAMP-BART assay (Gandelman et al., 2010). The extremely large amounts of DNA produced during the LAMP amplification lead to a high yield of PPI that can be converted into ATP by the enzyme ATP
sulfurylase. The produced ATP molecules together with the co-factor luciferin are used in the reaction catalysed by firefly luciferase in which light is generated (Gandelman et al., 2010). The emitted light can be monitored with the progress of LAMP amplification, showing a signature light output peak, and the time for this peak to appear is inversely proportional to the concentration of ATP, and hence PPI, and consequently the starting template (Gandelman et al., 2010). So far, there is no report of this method being used for foodborne virus detection. Therefore, RT-LAMP-BART was investigated in the current research for the detection of HAV in food.

1.5.3.3. Qβ replicase reaction assay

1.5.3.3.1. Bacteriophage Qβ

The bacteriophage Qβ was first isolated from a sewer in Kyoto, Japan in the early 1960s (Loeb, 1960; Loeb & Zinder, 1961; Miyake et al., 1971; Weissmann, 1974). It is a lytic RNA bacteriophage that belongs to the Leviridae family and Allolevivirus genus that infects E. coli cells having F-pili (Van Regenmortel et al., 2000; Woody & Cliver, 1997). The size of the bacteriophage Qβ virion is between 21.3 – 29.4 nm (Simonet & Gantzer, 2006). The Qβ phage capsid consists of three different proteins, the coat protein, the A₁ protein, and the A₂ protein (Valegård et al., 1994). The main component is the coat protein. There is only one copy of A₂ protein and about five copies of the A₁ protein per virion (Weissmann et al., 1973). In total, the number of protein subunits in a capsid shell is 180 and these proteins are arranged in a \( T = 3 \) icosahedral quasi-symmetry shell that encloses a positive-sense ssRNA (Golmohammadi et al., 1996). The length of Qβ genome RNA is 4,217 nt, which has three open reading frames (ORFs) encoding four different proteins (Figure 1.4). From 5’ end, these are the protein A₂,
coat protein and read-through protein A₁, and β subunit of RNA-dependent RNA polymerase, respectively (Schuppli et al., 1994). Protein A₂ is called maturation protein or lysis protein, because it functions in both the processes of phage capsid maturation and host cell lysis (Karnik & Billeter, 1983; Winter & Gold, 1983). Protein A₂ is essential in the attachment of bacteriophage Qβ to the pili of the host cell (García-Villada & Drake, 2012; Vallenweider et al., 1994; Weissmann et al., 1973). Protein A₁ is called read-through protein, because it is transcribed by read-through of the UGA-termination site at the end of the coat cistron (Hofstetter et al., 1974). Protein A₁ is essential for maintaining the infectivity of phage particles (Brown et al., 2009). Coat protein is required for the phage capsid assembly (Takamatsu & Iso, 1982). The RNA-dependent RNA polymerase, also called Qβ replicase, is indispensable for the replication of the phage genome (Inokuchi et al., 1994; Mills et al., 1988; Moore et al., 1971). The life cycle of Qβ phage can be summarized as follows: (1) the phage attaches to the F-pili of host E. coli cell and the RNA genome enters the host cytoplasm; (2) cellular components translate the β subunit of Qβ replicase; (3) Qβ replicase copies (+)-strand RNA genome to produce a (-)-strand RNA, which in turn is used as a template for the production of more (+)-strands; (4) (+)-strands serve as templates for the production of phage proteins; (5) phage proteins and (+)-strand RNAs assemble phage virions and phage particles are released from the host cell by cell lysis (García-Villada & Drake, 2012).

Figure 1.4. Genetic map of the phage Qβ (Klovins et al., 1998; Vollenweider et al., 1976).
1.5.3.3.2. Subunits of Qβ replicase

Qβ replicase is a heterotetrameric protein complex with a molecular mass of 215 KDa (Gunasekaran et al., 2013). It was first purified from E. coli cells infected with Qβ phage in 1965 (Spiegelman et al., 1965). The enzyme is composed of four subunits designated as subunit α, β, γ, and δ, respectively, by Kamen et al. (1972). Three subunits, α, γ, and δ, are supplied by the host. The subunit α is the 30S ribosomal protein S1 (70 KDa). The subunit γ and δ are translational elongation factors EF-Ts (45 KDa) and EF-Tu (35 KDa), respectively. The β subunit (65 KDa) is the only protein encoded by the phage RNA (Blumenthal & Carmichael, 1979; Lindner et al., 1991; Sumpner & Luce, 1975; Young & Blumenthal, 1975).

EF-Tu and EF-Ts are firmly bound with the β subunit and form with it a stoichiometric complex, while S1 is bound loosely and is not present in every molecule of the Qβ replicase (Blumenthal & Carmichael, 1979; Vasiliev et al., 2010). Each subunit plays a specific role in RNA replication (Blumenthal & Carmichael, 1979). *In vivo*, Qβ replicase synthesizes (−)-strand RNA by reading (+)-strand RNA of Qβ genome. In the next replication round, both of the strands serve as templates for the synthesis of their complementary copies, and so on. Thus, the number of RNA copies doubles in each round, and increases exponentially as long as the replicase remains in molar excess over RNA. Qβ replicase only replicates ssRNA but not dsRNA (Axelrod et al., 1991; Brown & Gold, 1995). The function of subunit α, 30S ribosomal protein S1, is to stimulate the *de novo* initiation (without an RNA primer) of RNA replication and promote the release of the product strand in a single-stranded form, thereby ensuring RNA amplified exponentially (Vasilyev et al., 2013). The β subunit is responsible for recognizing Qβ phage RNA and helping Qβ replicase distinguish its own RNA and other RNAs. With the deletion of β subunit, Qβ replicase will lose its RNA polymerizing activity.
and template specificity (Inokuchi & Kajitani, 1997). The subunit γ, EF-Tu, functions as a replication cofactor for RNA polymerization. It assists in the initiation of RNA replication (Preuss et al., 1997), separation of the dsRNA, and separation of RNA with β subunit, and acts as a modulator for progressive RNA polymerization at the RNA elongation stages (Takeshita & Tomita, 2012). The subunit δ, EF-Ts, forms the complex with EF-Tu and is able to fix the structure of EF-Tu, thus making the domain of EF-Tu available for interactions with the β subunit and ensuring the β subunit is in its proper conformation (Blumenthal & Carmichael, 1979; Fukano et al., 2002; Preuss et al., 1997).

1.5.3.3.3. Template specificity of Qβ replicase

Qβ replicase displays high template specificity during infection, replicating the phage RNA (4,217 nt) while ignoring the extraordinary excess of bacterial RNA in the host cell. Such specificity is observed in vitro as well (Brown & Gold, 1995; Klovins et al., 1998). Qβ replicase can utilize a certain number of short-chained RNA (30 – 250 nt) for replication (Preuss et al., 1997). There have been a number of sequence-identified small RNAs efficiently replicated by Qβ replicase. They are 6s RNA (130 nt) (Banerjee et al., 1969), which is generated in vivo and “variant RNAs” synthesized in vitro, for example, nanovariant RNA WSI (91 nt) (Schaffner et al., 1977), minivariant RNA MV-1 (151 nt) (Kacian et al., 1971), microvariant RNA (114 nt) (Mills et al., 1975), midivariant RNA MDV-1 (218 nt) (Kacian et al., 1972), salt-resistant variant RNA SV-11 (113 nt) (Biebricher et al., 1982), RQ (replicable by Qβ replicase) 120 (120 nt) (Munishkin et al., 1988), and RQ 135 (135 nt) (Munishkin et al., 1991). These RNAs do not have significant sequence similarity. However, they share some common characteristics: (1) a GGG at the 5’ terminus and CCCA at the 3’ terminus.
(Chetverin et al., 1991), (2) the ability to form a stable hairpin loop near the 5’ end of the template (Schuppli et al., 1994), (3) the potential to involve many nucleotides in intramolecular base-paring (Schuppli et al., 1994), (4) a polypyrimidine-rich region of at least eight nucleotides exists for Qβ replicase binding (Brown & Gold, 1995). The mechanism of specific amplification of these RNAs by Qβ replicase is that each of them contains a structural feature, which enables the binding site and the active center of Qβ replicase together (Barrera et al., 1993; Kacian et al., 1971; Lindner et al., 1991). Qβ replicase binds to an internal site of RNA but initiates the replication from 3’ terminus. Thus, if a RNA cannot fold in a way that brings together the internal binding site and 3’ end together, the RNA will not be the template of Qβ replicase (Jacobson, 1991; Jacobson & Zuker, 1993).

These small RNAs come from Darwinian natural selection (Kacian et al., 1972; Moody, et al., 1994). The enzymes involved in RNA amplification have rather high error rates, because proof-reading and other error repair mechanisms are lacking (Rohde et al., 1995). Thus, RNA mutations occur spontaneously in its amplification and RNA evolves towards the favorable direction by natural selection forces. When Qβ RNA is amplified by Qβ replicase for many rounds, the rate of RNA synthesis increases and variants of short RNAs arise. The smaller the RNA chain, the shorter the time required for its synthesis. The replication rate of smaller RNAs is reported to be 15 times faster than the complete Qβ RNA (Mills et al., 1967).
Figure 1.5. Secondary structure of MDV-1. The arrow points the position for inserting heterologous sequences.

Among these short RNAs, MDV-1 (218 nt) has been studied intensively and utilized as a specific reporter probe in nucleic acid hybridization assays. The nucleotide sequence of both a 35-nt sequence at the 3' end of MDV-1 and a 46-nt sequence near the center of the MDV-1 sequence are almost identical to sequences at the 3' end and at an internal region of Qβ RNA (Inokuchi & Kajitani, 1997; Moody et al., 1994). The binding site for Qβ replicase is located near the middle of MDV-1 RNA and the initiation site is at the 3' end (Inokuchi & Kajitani, 1997; Nishihara et al., 1983). A heterologous RNA sequence can be inserted into the specific site of MDV-1 without disrupting the native secondary structure of MDV-1 (Figure 1.5), producing a new recombinant RNA that can still be amplified by Qβ replicase (Axelrod et al., 1991). Therefore, if the inserted RNA sequence can hybridize with a target specifically, the
resultant recombinant RNA can be used as a reporter probe in the Qβ replicase reaction assay (Lizardi et al., 1988).

1.5.3.3.4. Qβ replicase reaction assay

Qβ replicase reaction assay is based on nucleic acid hybridization and Qβ replicase amplification. Apart from the reporter probe, there is another capture probe involved in the assay. The capture probe is a short, ssDNA sequence complementary to the target, with its 5’ end biotin-labeled, which can enable a capture probe to bind strongly to streptavidin that is covalently linked to paramagnetic particles (Tyagi et al., 1996). In the Qβ replicase reaction assay, sufficient amounts of reporter probes and capture probes are added to the sample to hybridize with target RNA. The hybrids are then captured by streptavidin-linked paramagnetic particles, and non-hybridized reporter probes are washed away. After that, the reporter probes are released from the hybrids and then are exponentially replicated by Qβ replicase. In the presence of a fluorescent RNA binding dye such as SYBR green II (Bansho et al., 2012; Hosoda et al., 2007), propidium iodide (Burg et al., 1995), or ethidium bromide (Kramer & Lizardi, 1989), the amplification can be monitored in real-time by a fluorometer (Hosoda et al., 2007). This assay is less sensitive to inhibitors than PCR and does not need a thermal cycler since the reaction occurs at 37°C (An et al., 1995). This assay is also very rapid, with one molecule of MDV-1 amplified a million-fold within 12 min (Chu et al., 1986; Wu et al., 1992). Because of these attributes, this assay has been used for detecting pathogens, such as Mycobacterium tuberculosis (An et al., 1995; Shah et al., 1995a, 1995b), Chlamydia trachomatis (Shah et al., 1994), Mycobacterium avium complex, Mycoplasma pneumoniae, Pneumocystis carinii, Legionella pneumophila (Stone et al., 1996), and human immune
deficiency virus (HIV) (Axelrod et al., 1991; Lizardi et al., 1988; Lomeli, Tyagi et al., 1989; Tyagi et al., 1996), in clinical samples and has been shown to be 10-fold more sensitive than PCR for detecting *Mycobacterium tuberculosis* in sputum samples (An et al., 1995). Thus, this method was evaluated in the current research for the detection of HAV.

1.6. Objectives of this thesis

As stated above, the problems for the concentration and detection of low contamination level of viruses in food still exist. Rapid, easy-to-perform, and cost-effective methods are urgently required to improve the efficiency of foodborne virus detection. The purpose of this thesis is to develop novel methods for the concentration and detection of foodborne viruses, using HAV as a model virus. A Fe₃O₄ MNP-based method was developed in this thesis for the concentration of HAV from different food matrices. Isothermal amplification techniques, RT-LAMP-BART assay and Qβ replicase reaction assay, were also investigated to detect HAV. The methods developed in this research will provide directions to detect other viruses, bacteria, chemicals and other contaminants in foods and will ultimately improve food safety, reduce foodborne illnesses and economic loss. There were four objectives for this research. The first objective was to fabricate cationic Fe₃O₄ MNPs for the concentration of HAV from foods. This included:

1) Developing and optimizing the protocol for making cationic Fe₃O₄ MNPs;

2) Optimizing the reaction conditions of using the cationic MNPs to concentrate HAV from virus suspensions.
The second objective of this research was to compare different methods for the concentration of HAV from different food matrices. This included:

1) Developing the optimal procedure of using the cationic MNPs to concentrate HAV from green onions, strawberries, mussels, and milk;
2) Comparing the efficiency of PEG dialysis and cationic MNP capture method for the concentration of HAV from green onions, strawberries, mussels, and milk.

The third objective of this research was using RT-LAMP-BART assay to detect HAV concentrated from green onions, strawberries, mussels, and milk. This included:

1) Optimizing the reaction conditions of RT-LAMP-BART assay to improve the sensitivity;
2) Comparing the efficiency of RT-LAMP-BART and real-time RT-PCR to detect HAV concentrated from different foods.

The fourth objective of this research was using Qβ replicase reaction assay to detect HAV concentrated from green onions, strawberries, mussels, and milk. This included:

1) Designing reporter probes used to hybridize with HAV genome and amplified by Qβ replicase;
2) Developing a protocol to remove non-hybridized reporter probes in the reaction to reduce background signal;
3) Comparing the efficiency of real-time RT-PCR and Qβ replicase reaction assay in detecting HAV in green onions, strawberries, mussels, and milk.
Chapter 2: FABRICATION OF CATIONIC MAGNETIC NANOPARTICLES FOR THE CONCENTRATION OF HEPATITIS A VIRUS

2.1. Introduction

An increase in the globalization of food trade in recent years has presented new challenges for food safety and resulted in an increased number of foodborne outbreaks (Hall et al., 2005). The growing burden of the foodborne outbreaks due to viral contamination of food has become a significant public health concern. Better laboratory detection techniques are required to identify the source of contamination. In comparison with bacterial pathogens, it is challenging to develop methods for the detection of viruses in food due to their rather low contamination level and most viruses are difficult or not able to be enriched by cell culture (Boxman et al., 2012; Felix-Valenzuela et al., 2012). Thus, prior to viral genome amplification by molecular biological detection methods, viral particles need to be separated and concentrated from contaminated foods to help enhance the efficiency of virus detection.

Currently, PEG precipitation, ultracentrifugation, ultrafiltration, positively- or negatively-charged membrane filtration, and immunomagnetic separation methods are commonly used to concentrate viruses from foodstuffs (Stals et al., 2012). However, the application of these methods is limited by some disadvantages inherent in each of them. For example, the ultrafiltration method requires viral eluates free of food components; the ultracentrifugation method needs an expensive ultracentrifuge and specialized personne; the immunomagnetic separation method is only suitable for processing samples with small volume due to the high...
price of the antibodies; and the PEG precipitation method is time-consuming (Stals et al., 2012). As to the charged-membrane filtration method, a considerable number of viral particles passed through the pores of membranes instead of being trapped by the electrostatic force (Di Pasquale et al., 2010). Hence, it is meaningful to develop a new method to concentrate viruses from food samples.

Recently, there have been many studies focused on iron oxide (Fe$_3$O$_4$) MNPs, due to their unique physiochemical properties such as high surface-to-volume ratio, rapid diffusion, good dispersability, and many unique size-dependent qualities (Yang et al., 2008). MNPs have been used to concentrate foodborne bacterial pathogens, such as E. coli (Cheng et al., 2009; Ravindranath et al., 2009; Varshney et al., 2005), S. Typhimurium (Ravindranath et al., 2009), and L. monocytogenes (Amagliani et al., 2006; Yang et al., 2007), and have demonstrated higher efficiency than microbeads. However, there are no reports about the applications of MNPs in capturing viruses in foods. Therefore, the use of Fe$_3$O$_4$ MNPs (20 – 30 nm diameter) to concentrate viruses from food samples was studied in our work. HAV has been always a prevalent viral pathogen in contaminated vegetables, fruits, shellfish, and ready-to-eat foods. Therefore, in our study, HAV was used as a model foodborne virus to build the MNP capture method.

The new antigenic variants of HAV strains make the virus escap from capture by currently available anti-HAV antibodies (Sánchez et al., 2002). Cationic Fe$_3$O$_4$ MNPs produced by coating -NH$_2$ groups or a unique basic food protein, protamine, onto the surface of MNPs were investigated in the current study. The pI of HAV is 2.8 (Michen & Graule, 2010), and
thus it is expected that, at pH above 2.8, HAV particles are negatively charged and could form a complex with positively charged MNPs by electrostatic attractive force. The complex can be easily separated from the food using a magnet.

The purpose of the work described in this chapter was to synthesize surface modified iron oxide MNPs (20 – 30 nm diameter) and optimize the reaction conditions to allow modified MNPs to recover HAV from glycine buffer containing the virus, since glycine buffer with pH of 9.0 – 10.5 has been widely used to rinse HAV particles from contaminated foods and has proved to be efficient (Stals et al., 2012).

2.2. Materials and methods

2.2.1. Production of NH$_2$-coated Fe$_3$O$_4$ MNPs

Iron oxide (Fe$_3$O$_4$) MNPs were purchased from Sigma-Aldrich Co. (Cat. No. 637106, Oakville, ON, Canada). Different methods for coating -NH$_2$ groups on the surface of MNPs have been described in the literature (Bruce & Sen, 2005; Chang & Adriaens, 2006; Kralj et al., 2011). In these methods, different solvents (i.e., ethanol or water) and MNP suspending approaches (i.e., sonication or shaking) were used. The solvents and MNP suspending methods were optimized in our research. The procedure of four different protocols was as follows:

**Method A:** One hundred mg of Fe$_3$O$_4$ MNPs were added to 23.7 mL of Milli-Q water and mixed by vortex and sonication at 40 kHz in a bath sonicator (FS20, Fisher Scientific, Ottawa, ON, Canada) until the suspension was completely homogenized. Then, 1.3 mL of 3-
aminopropyltriethoxysilane (APTES, Cat. No. A3648, Sigma-Aldrich, Oakville, ON, Canada) were added to the homogenized suspension, which was then shaken at 250 rpm for 24 h at 50°C. After that, the MNPs were recovered using a permanent magnet with a surface magnetization of 13,000 gauss (Cat. No. 44207-20, Indigo Instruments, Waterloo, ON, Canada), followed by thoroughly washing the MNPs with Milli-Q water for five times to remove unbound APTES. Finally, the NH₂-coated MNPs (NMNPs) were resuspended in 10 mL of Milli-Q water and stored at 4°C.

**Method B:** One hundred mg of Fe₃O₄ MNPs were added to 23.7 mL of 90% (v/v) ethanol and vortexed and sonicated at 40 kHz until the suspension was completely homogenized. Then, 1.3 mL of APTES were added to the homogenized suspension, which was subsequently shaken at 250 rpm for 24 h at 50 °C. The NMNPs were recovered and washed with Milli Q water as described in Method A and stored at 4°C.

**Method C:** One hundred mg of Fe₃O₄ MNPs were added to 23.7 mL of Milli Q water. The mixture was homogenized by vortex and sonication as described in Method A. Then, 1.3 mL of APTES were added to the MNP suspension, followed by sonication at 40 kHz for 10 h at 50°C. Finally, the NMNPs were recovered, washed, and stored in 10 mL of Milli-Q water at 4°C as described in Method A.

**Method D:** One hundred mg of Fe₃O₄ MNPs were mixed with 23.7 mL of 90% (v/v) ethanol and 1.3 mL of APTES. The mixture was homogenized and then sonicated at 40 kHz for 10 h
at 50°C as described in Method C. The NMNPs were recovered, washed, and stored in 10 mL of Milli-Q water at 4°C.

2.2.2. Production of COOH-coated Fe₃O₄ MNPs

Carboxyl groups were coated on the surface of NMNPs according to Zhao et al. (2004). After being washed three times with N, N-dimethylformamide (DMF) (Cat. No. D4551, Sigma-Aldrich, Oakville, ON, Canada), NMNPs were suspended in 25 mL of DMF containing 10% (w/v) succinic anhydride (Cat. No. 239690, Sigma-Aldrich, Oakville, ON, Canada). The chemical reaction was performed at 50°C under nitrogen gas with shaking at 250 rpm for 6 h. The nanoparticles were harvested using the magnet and washed thoroughly with Milli-Q water for five times to remove unbound reactants. The COOH-coated MNPs (CMNPs) were re-suspended in 10 mL of Milli-Q water and stored at 4°C.

2.2.3. Production of protamine-coated Fe₃O₄ MNPs

Protamine from salmon (mol. wt. 5100, Cat. No. P4020, Sigma-Aldrich, Oakville, ON, Canada) was coated on the surface of the CMNPs by EDC/NHS coupling chemistry (Ravindranath et al., 2009; Zhao et al., 2004). EDC (1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride) (Cat. No. PI-22980) and NHS (N-hydroxysuccinimide) (Cat. No. 130672) were purchased from Fisher Scientific Inc. (Ottawa, ON, Canada) and Sigma-Aldrich Co. (Oakville, ON, Canada), respectively. One mL of CMNPs was washed with 0.1 M MES (4-Morpholineethanesulfonic acid) (Cat. No. M8250, Sigma-Aldrich, Oakville, ON, Canada) buffer (pH 6.0) for three times, and was then activated with 0.4 M EDC/0.1 M NHS prepared in 0.1 M MES buffer (pH 6.0) by rotation at 30 rpm for 40 min at room temperature. The MNPs were magnetically recovered, washed three times with 0.1 M PBS (pH 7.5), and
subsequently mixed with 2 mL of 10 mg/mL protamine in 0.1 M PBS (pH 7.5). The reaction mixture was rotated at 30 rpm and 25°C for 24 h, followed by washing the MNPs three times with 0.1 M PBS (pH 7.5), twice with 3 M NaCl, once with 10 mM HCl, and three times with Milli-Q water. Finally, the protamine-coated MNPs (PMNPs) were suspended in 1 mL of Milli-Q water and stored at 4°C.

2.2.4. Characterization of chemically modified Fe₃O₄ MNPs

2.2.4.1. Fourier transform infrared spectroscopy (FTIR)

The Fe₃O₄ MNPs coated with -NH₂ groups, -COOH groups, or protamine were scanned using a Diglab FTS-7000 Fourier transform infrared spectrometer (Bio-Rad, Randolph, MA, USA) equipped with a DTGS (deuterated triglycine sulfate) detector and a Golden Gate single reflection diamond ATR (attenuated total reflectance) accessory. The sample for FTIR spectroscopy was vacuum-dried at 40°C. The spectra were collected from 800 cm⁻¹ to 4000 cm⁻¹ at 4 cm⁻¹ resolution with 64 co-added scans per spectrum.

2.2.4.2. Zeta potential analysis

Zeta potentials of Fe₃O₄ MNPs were measured using a Malvern Zetasizer Nano ZS instrument (Malvern Instruments Ltd. Malvern, Worcestershire, United Kingdom). After equilibrating the samples for 24 h at room temperature, the zeta potentials of the MNPs were tested in aqueous suspension at pH of 3.0 – 11.0 adjusted by 0.1 M HCl or NaOH.

2.2.4.3. Transmission electron microscopy (TEM)

The morphology of Fe₃O₄ MNPs with protamine coating was observed by a LEO 912 AB energy filtered transmission electron microscope (Carl Zeiss Inc. Oberkochen, Germany)
operating at 100 kV. The sample preparation for TEM analysis included pipetting 5 µL of MNP suspension onto a formvar and carbon foil covered copper grid (200 mesh). The excess liquid was removed by blotting with filter paper, and then the copper grid was floated on a drop of 2% (w/v) uranyl acetate for 30 s. Images were captured with an Olympus/SIS Cantega 2K digital camera using Olympus/SIS item software.

2.2.5. HAV propagation

HAV strain HM175/24A, a cytopathic clone of strain HM175 adapted to cell culture, was obtained from the culture collection of the Canadian Research Institute for Food Safety (CRIFS) and propagated in fetal rhesus monkey kidney cells (FRhk-4), as described by Morales-Rayas et al. (2010). Briefly, FRhk-4 cells were cultured in Dulbecco's Modified Eagle Medium (DMEM, Cat. No. 12800017, Life Technologies, Burlington, ON, Canada) supplemented with 1% (v/v) streptomycin/penicillin (Cat. No. 15140-122, Life Technologies, Burlington, ON, Canada) and 10% (v/v) heat-inactivated fetal bovine serum (FBS, Cat. No. 16140-063, Life Technologies, Burlington, ON, Canada) in a 75 cm² tissue culture flask (Cat. No. 430720, Corning, Tewksbury, MA, USA). Cells were incubated at 37°C and 5% CO₂ for 2 days until confluent growth was achieved. The confluent monolayer was then washed once with PBS (pH 7.4, Cat. No. 10010031, Life Technologies, Burlington, ON, Canada) warmed at 37°C. Five mL of DMEM and 200 µL of viral stock were inoculated on the surface of the monolayer with a MOI (multiplicity of infection) of 0.1. The flask was incubated at 37°C for 1.5 h, with gentle shaking every 10 min to distribute the inoculum. After 1.5 h, the inoculum was removed and 8 mL of DMEM supplemented with 2% (v/v) FBS and 1% (v/v) streptomycin/penicillin were added to the flask. Cells were incubated for 8 days until 80% of
them showed a cytopathic effect. The viruses were released from the cells by three freeze-thaw cycles, and cell debris was removed by centrifugation at $1,600 \times g$ for 10 min using a Beckman Coulter Allegra 21 centrifuge with an S4180 rotor (Mississauga, ON, Canada). The supernatant was then filtered through a Millex 0.22 µm (GV) low protein binding filter (Millipore Ltd. Etobicoke, ON, Canada) and dispensed into cryogenic vials in 1-mL aliquots and stored at -80°C.

2.2.6. HAV plaque assay

Viral stock was quantified by plaque assay as described by Cromeans et al., (1987). Briefly, FRhk-4 cells were inoculated into 6-well tissue culture plates (Cat. No. C3506, Corning, Tewksbury, MA, USA) with $5 \times 10^5$ cells dispensed in each well. The plates were incubated at 37°C and 5% CO$_2$ for 24 h. After washing the monolayer with PBS (pH 7.4), 500 µL of 10-fold serial dilutions of viral stock in DMEM were inoculated onto the plates, with each dilution tested in triplicate. The plate was incubated for 1.5 h with gentle shaking every 10 min. The inoculum was removed and 3 mL of overlay medium, a mixture of 1.5 mL of 1.2% (w/v) SeaKem LE Agarose (Cat. No. S0002, Cambrex Bio Science, Rockland, ME, USA) containing 26 mM MgCl$_2$ and 1.5 mL of 2 × DMEM supplemented with 4% (v/v) FBS, 2% (v/v) streptomycin/penicillin, were added to each well. The plate was incubated for 10 days. Then, after fixing with 10% formalin (Cat. No. 23245684, Fisher Scientific, Ottawa, ON, Canada) for 24 h, the overlay was removed, and the monolayer was stained with 0.1% (w/v) crystal violet (prepared in 0.85% saline, w/v) for 20 min. The plaques were counted after the removal of crystal violet.
2.2.7. Real-time RT-PCR of HAV

Viral RNA was extracted from 200 µL of viral stock using the QIAamp MinElute Virus Spin Kit (Cat. No. 57704, Qiagen, Toronto, ON, Canada) according to the manufacturer’s instructions. Briefly, 200 µL of virus were mixed with 25 µL of Qiagen protease and 200 µL of buffer AL in the Kit. The mixture was incubated at 56°C for 15 min, followed by the addition of 250 µL of ethanol (100%) and incubation at room temperature for 5 min. The sample was loaded onto the QIAamp MinElute column and centrifuged at 6,000 × g for 1 min using a Beckman Coulter Allegra 21 centrifuge with an F2402H rotor (Mississauga, ON, Canada). After washing the column sequently with 500 µL of buffer AW1, AW2, and ethanol (100%) by centrifugation at 6,000 × g for 1 min, the column was dried by centrifugation at 20,000 × g for 3 min. Finally, 40 µL of RNase-free water (Cat. No. AM9937, Life Technologies, Burlington, ON, Canada) were used to elute RNA from the column and the eluted RNA was stored at -80°C.

Ten µL of RNA were reverse transcribed to cDNA using High-capacity cDNA Reverse Transcription Kit (Cat. No. 4374966, Life Technologies, Burlington, ON, Canada) according to the manufacturer’s instructions. Briefly, the RNA was mixed with 2 µL of 10 × RT buffer, 2 µL of RT random primers, 0.8 µL of 25 × dNTP mix, 1 µL of multiscribe reverse transcriptase, 1 µL of RNase inhibitor, and 3.2 µL of RNase-free water in a 20 µL reaction system. The reagents were mixed on ice. The mixture was incubated using an Applied Biosystems GeneAmp PCR system 9700 (Life Technologies, Burlington, ON, Canada) at 25°C for 10 min, 37°C for 120 min, 85°C for 5 min, and then at 4°C until PCR analysis.
Real-time PCR was performed with an ABI 7900HT sequence detection system (Life Technologies, Burlington, ON, Canada) using primers and probe: 5’ATAGGGTAACAGCGCGGATAT3’ (forward), 5’CTCAATGCATCCACTGGATGAG 3’ (reverse) and 5’FAM-CCATTCAACGCGGGAGG-MGB3’ (Taqman probe) (Gardner et al., 2003). The primers and probes were mixed with the PCR mixture as follows: 1 × Absolute QPCR ROX mix (Cat. No. AB1138A, Fisher Scientific, Ottawa, ON, Canada), 700 nM of each primer, and 250 nM of probe in a 25 µL reaction system. The amplification was performed with enzyme activation for 15 min at 95°C, followed by 40 cycles of 15 s at 95°C, 60 s at 60°C. A standard curve was constructed using 10-fold serial dilutions of cDNA. The amplification efficiency was calculated using the formula E=10^{-1/slope}-1 (Klein et al., 1999). Each cDNA was measured in triplicate.

2.2.8. Optimization of MNP capture conditions

In order to optimize the HAV concentration method using cationic MNPs, the recovery efficiency of HAV from 50 mL of viral suspensions was examined at different pH, NaCl concentrations and MNP concentrations.

**Effect of pH:** Glycine buffers (0.05 M glycine, 0.14 M NaCl, 0.2% (v/v) Tween 20) with pH of 4.5, 6.0, 7.5, and 9.0 were prepared in the following way: pH was first adjusted to 9.0 using 1 M NaOH, and then adjusted to 7.5, 6.0, and 4.5, respectively, using 1 M HCl. One hundred µL of virus suspension (3.8 × 10^5 PFU/mL) were inoculated into 50 mL of glycine buffer with different pH values. One hundred µL of cationic MNPs were added into the reaction system to capture the virus. The mixture was rotated at 10 rpm at room temperature for 20 min, followed
by magnetic capture for 30 min. The supernatant was discarded and 220 µL of HCl (pH 1) were added to the tube to release the virus from cationic MNPs after incubation for 15 min. After magnetic capture for 5 min, the supernatant was transferred to a 1.5 mL centrifuge tube and was neutralized with NaOH solution (1 M). The viral particles captured at each pH were quantified using real-time RT-PCR reaction. The recovery efficiency of MNPs was determined by the equation: recovery rate = (total viral particles captured / total viral particles input in buffer before separation) × 100% (Morales-Rayas et al., 2010). Both NMNPs and PMNPs were used to recover HAV in this test. The recovery efficiency of HAV using these two different MNPs was compared. Each experiment was carried out in three independent replicates.

**Effect of NaCl:** Fifty mL of glycine buffers (0.05 M glycine, 0.2% (v/v) Tween 20, pH 9.0) containing NaCl at concentrations of 0.14, 0.28, 0.56, or 1 M were inoculated with 100 µL of viral suspension (3.8 × 10⁵ PFU/mL) and 100 µL of MNPs. The recovery efficiency of HAV in each buffer was evaluated using the aforementioned method in three independent experiments.

**Effect of MNP concentration:** Different volumes (50, 100, 200, or 300 µL) of PMNPs were added to 50 mL of glycine buffers (0.05 M glycine, 0.14 M NaCl, 0.2% (v/v) Tween 20, pH 9.0) inoculated with 100 µL of viral suspension at different concentrations (3.8 × 10⁶, 3.8 × 10⁴, 3.8 × 10², or 3.8 × 10¹ PFU/mL). The recovery efficiencies of HAV using different concentrations of PMNPs were evaluated as previously described in two independent replicates.
2.2.9. Statistical analysis

The recovery rates of HAV using MNP capture method performed under different pH, NaCl concentrations, and MNP concentrations were expressed as the mean ± standard deviation. Statistical analysis of the means was performed using one-way analysis of variance (ANOVA) and subsequent Tukey's test with IBM SPSS Statistics (version 21; IBM Corporation, New York, USA). In all cases, $P$-value < 0.05 indicated a significant difference.

2.3. Results and discussion

2.3.1. Zeta potential analysis of chemically modified Fe$_3$O$_4$ MNPs

The efficiency of -NH$_2$ group coating was followed by measurement of zeta potential of MNPs in the aqueous suspension. The electrophoretic mobility of the MNPs was measured at different pH (3.0 – 11.0). The changes in the zeta potential of the NMNPs prepared using different methods are shown in Figure 2.1. For each sample, with the increase of pH, zeta potentials decreased. The pI is the pH at which MNPs have zero zeta potential. The positive surface charges provided by -NH$_2$ groups results in a shift of the pI to higher pH values. The MNPs produced by shaking at 50°C for 24 h in water showed the highest zeta potential values at each tested pH and the most significant increase of pI, indicating the greatest surface concentration of -NH$_2$ groups.
Figure 2.1. Zeta potentials of NH$_2$-coated MNPs produced under different conditions. Each value is the mean of five replicates. Bars show standard deviation.

For the -COOH coating, the -NH$_2$ groups on the surface of NMNPs were reacted with succinic anhydride by attacking the carbonyl C atoms of the succinic anhydride molecule to form amide bonds, resulting in free terminal -COOH groups from the elongated chains. Fig. 2.2 shows the zeta potential of MNPs in aqueous suspension with different pH (3.0 – 11.0). The MNPs were first coated with -NH$_2$ groups using different methods and subsequently reacted with succinic anhydride under the same conditions. Compared with NMNPs (Figure 2.1), the pI of CMNPs shifted to lower values due to the acidic character of the negatively charged -COOH groups. The CMNPs produced from NMNPs prepared by shaking at 50°C for 24 h in water demonstrated the lowest zeta potential values at each pH and the most significant decrease of pI, indicating the greatest surface concentration of -COOH groups. This result further proved that shaking in water was necessary for -NH$_2$ group coating.
Figure 2.2. Zeta potentials of COOH-coated MNPs produced under different conditions. Each value is the mean of five replicates. Bars show standard deviation.

For the coating of protamine, the surface -COOH groups of CMNPs were activated by EDC to form an amine-reactive O-acyl-isourea intermediate that could react with the -NH₂ groups of protamine to form an amide bond (Grabarek & Gergely, 1990). The role of NHS used in the experiment is to protect the intermediate that is unstable in aqueous solutions (Staros, Wright, & Swingle, 1986). Zeta potentials of PMNPs measured at different pH (3.0 – 11.0) are shown in Figure 2.3. With the increase of pH, zeta potentials decreased. MNPs without coating had a pI of around 8.0. Coating -NH₂ groups on the surface of the MNPs resulted in an increase of the pI of the MNPs to around 10.0. When the surface of the MNPs was further modified with -COOH groups, the pI shifted to about 5.0. The pI of PMNPs was higher than 10.3. When the pH was lower than 9.0, both PMNPs and NMNPs were positively charged. At a given pH, the zeta potential of PMNPs was higher than that of NMNPs, indicating that the surface charge of the former was higher than that of the latter. The reaction time used for protein cross-linking
varied greatly in published protocols (Cheng et al., 2009; Varshney et al., 2005; Yang et al., 2007; Zhao et al., 2004). We extended the reaction time from 4 h to 24 h and found that the zeta potentials of PMNPs were increased at given pH values, indicating higher coating efficiency with longer reaction time (data not shown).

Figure 2.3. Zeta potentials of Fe₃O₄ MNPs with different coatings. Each value is the mean of five replicates. Bars show standard deviation.

2.3.2. FTIR analysis of chemically modified Fe₃O₄ MNPs

FTIR spectra in the range from 800 to 4000 cm⁻¹ were collected for Fe₃O₄ MNPs without and with chemical modifications (Figure 2.4). The NMNPs exhibited a strong band at 990 cm⁻¹, which was due to the asymmetric stretching vibration of Si-O-Si (Das et al., 2008; Lapin & Chabal, 2009; Pasternack et al., 2008). Peaks at 2925 and 2857 cm⁻¹ were caused by the asymmetric and symmetric stretching vibration, respectively, of -CH₂ groups (Das et al., 2008; Pasternack et al., 2008; Zhang et al., 2002). These results indicated the success of -NH₂ group
coating. The low-intensity bands at 1300 – 1600 cm\(^{-1}\) were assigned to both associated and free -NH\(_2\) groups, confirming the successful coating of MNPs with -NH\(_2\) groups (Zhang et al., 2002). The intensity of the bands related to APTES was relatively weak, in accordance with the small proportion of the surface layer of APTES compared to the bulk of reagent, which was in good agreement with previous reports (Bruce & Sen, 2005; Kouassi et al., 2005).

Surface modification of the NMNPs with succinic anhydride led to the formation of COOH-terminated MNPs. The band at 1648 cm\(^{-1}\) was generated by the C=O stretching vibration of Amide I. The band at 1545 cm\(^{-1}\) resulted from the N-H bending vibration of Amide II (Kim et al., 2010). The coating of -COOH groups was further confirmed by the existence of the stretching vibrational mode of -COOH at 1710 cm\(^{-1}\) and symmetric stretching vibration mode of carboxylate (COO-) at 1405 cm\(^{-1}\) (Frey & Corn, 1996).

The typical vibration mode of amide bonds also appeared in the spectrum of PMNPs. The characteristic absorption peak from symmetric stretching vibration of NH\(_3^+\) in protamine was found at 3200 cm\(^{-1}\) (Fukushima et al., 2011), indicating that protamine was successfully coated on the surface of MNPs.

Toluene was used by Chang & Adriaens (2006) for the coating of MNPs with -NH\(_2\) groups. When toluene was used in the current experiment, strong bands at 2925 and 2857 cm\(^{-1}\) were found in the FTIR spectrum of MNPs even when APTES was not present in the solvent. Additionally the band at 1710 cm\(^{-1}\) in the spectrum of COOH-coated MNPs was not found, indicating the efficiency of -COOH group and further -NH\(_2\) group coating was low. Additional
concern is raised by the fact that toluene is a hazardous chemical, causing unconsciousness and even death (http://en.wikipedia.org/wiki/Toluene). These findings coupled with its hazardous nature resulted in toluene not being used for the surface modification of MNPs. FTIR analysis of the MNPs produced with different protocols suggested that the related band intensity of NMNPs prepared by shaking in water at 50°C for 24 h was stronger than that from other protocols (data not shown), further suggesting that the highest surface concentration of -NH₂ groups was from Method A (Materials and methods). These results were consistent with those obtained using zeta potential analysis.

Figure 2.4. FTIR spectra of Fe₃O₄ MNPs with different coatings.
2.3.3. TEM analysis of protamine-coated MNPs

Both protamine-coated and uncoated MNPs were approximately spherical in shape (Figure 2.5). The diameter of the MNPs was less than 50 nm. Two regions with different densities were distinguished for the MNPs coated with protamine: the dense magnetite core and a less dense shell indicating a protamine layer. The thickness of the protamine layer was approximately 2 nm.

![TEM images of uncoated (A) and protamine-coated (B) Fe₃O₄ MNPs.](image)

2.3.4. HAV propagation and quantification

The titer of the propagated HAV HM175/24A determined by plaque assay was \(3.8 \times 10^6\) PFU/mL. A standard curve of real-time RT-PCR \((y = -3.372 x + 36.83, R^2 = 0.998)\), shown in Figure 2.6, was obtained by analyzing the Ct values of 10-fold serial dilutions of cDNA. The slope indicated a PCR efficiency of 97.9%. The limit of detection of the real-time RT-PCR assay was 3.8 PFU/mL.
Figure 2.6. Standard curve of real-time RT-PCR made by 10-fold serial dilutions of HAV cDNA using ABI 7900HT system. Each value is the mean of three independent replicates. Bars show standard deviation.

2.3.5. Recovery of HAV using MNPs under different pH, ionic concentrations, and MNP concentrations

Glycine buffer with pH 9.0 – 10.0 has demonstrated high recovery efficiency in eluting viruses from foods and has been widely used in collecting viral particles from various foods (Baert et al., 2007; Butot et al., 2007a; Casas et al., 2007; Dubois et al., 2002; Dubois et al., 2006; Guévremont et al., 2006; Rutjes et al., 2006; Suñén et al., 2004). Thus, in the current study, we selected glycine buffer for eluting HAV from foods and determined the optimal pH, ionic concentration, and concentration of MNPs for magnetic capture of HAV in the glycine buffer.

2.3.5.1. Optimization of pH

For both NMNPs and PMNPs, with the increase of pH, the recovery rate of HAV increased significantly ($P < 0.05$), with the highest recovery rate was obtained at pH 9.0 (Table 2.1). When pH was lower than 9.0, the recovery rate of HAV obtained by NMNPs was higher than
PMNPs. At pH 9.0, PMNPs showed a higher recovery rate than NMNPs. But the difference between the two MNPs under each pH was not significant \((P > 0.05)\). The pI of NMNPs and PMNPs was above pH 9.0 (Figure 2.3). The pI of HAV is around 2.8 (Michen & Graule, 2010; Tang et al., 1993). Thus, under all four pH conditions selected in our research, the charge of MNPs and HAV were opposite and electrostatic attraction force existed between the MNPs and virus. The highest recovery rate of HAV obtained at pH 9.0 indicated that the electrostatic attraction force between the MNPs and HAV particles was the strongest at this pH (Table 2.1). For preparation of buffers with different pH values, the initial pH was adjusted to 9.0 using NaOH, and then to the corresponding pH values using HCl. In this way, the lower the pH, the higher the ionic strength of the buffer. High level of ions in solution could compromise the electrostatic force, which led to the lowest recovery rate of HAV at pH 4.5. Therefore, the buffer with pH 9.0 was used in the following experiments.

<table>
<thead>
<tr>
<th>Capture pH</th>
<th>Recovery rate (%) of HAV by MNPs</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>NMNPs</td>
</tr>
<tr>
<td>4.5</td>
<td>13.8 ± 8.2\textsuperscript{a}</td>
</tr>
<tr>
<td>6.0</td>
<td>24.4 ± 10.5\textsuperscript{ab}</td>
</tr>
<tr>
<td>7.5</td>
<td>38.8 ± 10.7\textsuperscript{b}</td>
</tr>
<tr>
<td>9.0</td>
<td>41.2 ± 5.3\textsuperscript{b}</td>
</tr>
</tbody>
</table>

\textsuperscript{§}HAV concentration was \(3.8 \times 10^4\) PFU/50 mL and the volume of MNPs was 100 uL; Each value is the mean ± standard deviation \((n=3)\); Means within the same column with the same letter are not significantly different at the 5% confidence interval; Recovery rate = \((\text{total viral particles captured} \div \text{total viral particles input in buffer before separation}) \times 100\%\); PMNPs: protamine-coated magnetic nanoparticles; NMNPs: NH\(_2\)-coated magnetic nanoparticles.
2.3.5.2. Optimization of NaCl concentration

As is shown in Table 2.2, for both MNPs, HAV recovery rate decreased significantly with the increase of NaCl concentration ($P < 0.05$), with the highest recovery rate obtained with 0.14 M NaCl (Table 2.2). This result indicated that the electrostatic attraction force between the MNPs and viral particles was the strongest with 0.14 M NaCl. Increasing the ionic strength of a solution favors inhibition of the virus particles to MNPs by decreasing the effective radius of attractive electrostatic effects. The converse is also true. Lowering the ionic strength of the solution contributes to the enhancement of attraction between the virus and MNPs (Le Guyader & Atmar, 2008). For each NaCl concentration, the difference of recovery rate of HAV obtained with PMNPs and NMNPs was not significant ($P > 0.05$).

Table 2.2. Effect of NaCl concentration on HAV recovery rate using two cationic MNPs.

<table>
<thead>
<tr>
<th>NaCl concentration (M)</th>
<th>Recovery rate (%) of HAV by MNPs</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>NMNPs</td>
<td>PMNPs</td>
</tr>
<tr>
<td>0.14</td>
<td>33.6 ± 7.8$^a$</td>
<td>39.5 ± 5.9$^a$</td>
</tr>
<tr>
<td>0.28</td>
<td>23.5 ± 5.8$^{ab}$</td>
<td>27.4 ± 4.3$^{ab}$</td>
</tr>
<tr>
<td>0.56</td>
<td>20.3 ± 5.7$^{ab}$</td>
<td>22.0 ± 5.5$^{bc}$</td>
</tr>
<tr>
<td>1</td>
<td>15.1 ± 3.9$^b$</td>
<td>11.3 ± 3.0$^c$</td>
</tr>
</tbody>
</table>

HAV concentration was $3.8 \times 10^4$ PFU/50 mL and the volume of MNPs was 100 µL; Each value is the mean ± standard deviation (n=3); Means within the same column with the same letter are not significantly different at the 5% confidence interval; Recovery rate = (total viral particles captured / total viral particles input in buffer before separation) $\times$ 100%; PMNPs: protamine-coated magnetic nanoparticles; NMNPs: NH$_2$-coated magnetic nanoparticles.
2.3.5.3. Optimization of MNP concentration

For the inoculum level of $3.8 \times 10^0$, $3.8 \times 10^1$, and $3.8 \times 10^3$ PFU/50 mL of HAV, the concentration of MNPs significantly affected the recovery rate of HAV ($P < 0.05$), with the highest recovery rate was obtained with the use of 50 µL of protamine-coated MNPs (Table 2.3). With the increase of the concentration of PMNPs, the HAV recovery rate decreased implying that the enclosure of viral particles by large amount of MNPs might sterically hinder the release of captured viral particles. As for $3.8 \times 10^5$ PFU/50 mL of HAV suspension, the concentration of MNPs did not affect the viral recovery rate significantly ($P > 0.05$). Therefore, 50 µL of PMNPs were used in future experiments.

<table>
<thead>
<tr>
<th>Volume of MNP suspension (µL)</th>
<th>Recovery rate of HAV in suspension with different virus concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$10^0$</td>
</tr>
<tr>
<td>50</td>
<td>24.8 ± 5.9$^a$</td>
</tr>
<tr>
<td>100</td>
<td>12.6 ± 2.7$^{ab}$</td>
</tr>
<tr>
<td>200</td>
<td>13.3 ± 1.9$^{ab}$</td>
</tr>
<tr>
<td>300</td>
<td>8.5 ± 2.3$^b$</td>
</tr>
</tbody>
</table>

HAV concentrations ($3.8 \times 10^0$, $3.8 \times 10^1$, $3.8 \times 10^3$, or $3.8 \times 10^5$ PFU/50 mL) were tested; Each value is the mean ± standard deviation ($n = 2$); Means within the same column with the same letter are not significantly different at the 5% confidence interval; Recovery rate = (total viral particles captured / total viral particles input in buffer before separation) × 100%; All the viruses were captured at pH 9.0 and 0.14 M NaCl concentration.
2.4. Conclusions

Using a three-step chemical reaction method, protamine was covalently coated onto the surface of iron oxide (Fe₃O₄) MNPs. The successful conjugation of protamine was confirmed by a series of techniques including FTIR, zeta potential measurement, and TEM. The feasibility of two positively charged MNPs, PMNPs and NMNPs, for the recovery of HAV from 50 mL of viral suspensions (3.8 × 10⁰, 3.8 × 10¹, 3.8 × 10³, or 3.8 × 10⁵ PFU, 0.05 M glycine, 0.2% (v/v) Tween 20) was tested under different pH (4.5, 6, 7.5 and 9), NaCl concentrations (0.14, 0.28, 0.56, and 1M), and MNP concentrations (50, 100, 200, and 300 µL), with recovery efficiency determined by real-time RT-PCR. The optimal conditions for HAV recovery from 50 mL of viral suspension using cationic MNPs were pH 9, 0.14 M NaCl, and 50 µL of the MNPs. The recovery rate obtained under these conditions was between 24% and 49% in 70 min, depending on the initial viral load. The recovery efficiency of HAV from different food matrices using the two cationic MNPs will be described in the next Chapter.
Chapter 3: A COMPARISON OF DIFFERENT METHODS FOR THE
CONCENTRATION OF HEPATITIS A VIRUS FROM DIFFERENT
FOOD MATRICES

3.1. Introduction

Hepatitis A infection is the leading cause of viral hepatitis in many parts of the world. HAV is spread among humans by the fecal-oral route. HAV outbreaks are often associated with foods eaten raw or only slightly cooked, such as shellfish (Coelho et al., 2003; Croci et al., 2003; Goswami et al., 2002), fruits (Boxman et al., 2012), vegetables (Wheeler et al., 2005), ready-to-eat meat (Sun et al., 2012), and dairy products (Zaher et al., 2008). These foods maybe irrigated or cultivated with sewage-contaminated water or prepared by infected food handlers. In nearly 50% of reported hepatitis A cases, the mode and vehicles of viral transmission remain unidentified and infected food handlers may be involved in many cases of food contamination (Kukavica-Ibrulj et al., 2004). The infectious dose of HAV is between 10 and 100 particles (Yezli & Otter, 2011) and the contamination level of HAV in food samples is usually very low (Felix-Valenzuela et al., 2012). In the past, foods were rarely tested for viral contamination due to the lack of sensitive and reliable methods. A concentration step is necessary after elution of the virus from foods has been achieved, in order to aid subsequent molecular detection. Currently, there are no standard methods to efficiently concentrate viruses from different kinds of foods. The sensitivity of virus detection can be enhanced by a virus concentration method that is less time-consuming, easy-to-perform, cost effective, and with high yield of virus and less yield of food components.
It is difficult to obtain foods naturally contaminated with HAV because the long incubation period of HAV (15 – 50 days) results in the implicated foods unavailable when the source of HAV outbreaks is investigated (Castrodale et al., 2002). Thus, selecting food types and artificially contaminating the selected foods with HAV are necessary for food-borne HAV study. We selected mussels, milk, green onions, and strawberries in this study, because they represented several kinds of food matrices including complicated shellfish matrix, protein- and fat-rich dairy product, and carbohydrate- and water-rich fruit and vegetables. Mussels, green onions, and strawberries also represented solid food, for which glycine buffer needs to be used for the elution of viral particles. Milk represented liquid food that did not require elution. These four foods have been associated with HAV outbreaks (Elamri & Aouni, 2007; Manso & Romalde, 2013; Mesquita et al., 2011; Niu et al., 1992; Nordic outbreak investigation team, 2013; Wheeler et al., 2005).

In Chapter 2, we successfully fabricated NMNPs and PMNPs capable of efficiently recovering HAV from virus suspensions. The purpose of the work described in this chapter is to test the ability of the MNPs to recover HAV from green onions, strawberries, mussels, and milk artificially contaminated with HAV. The PEG dialysis method is cheap, simple, and easy-to-use for the concentration of protein (Degerli & Akpınar, 2001). Basically, this method has the potential to be used anywhere, as it does not require any sophisticated infrastructure. The PEG dialysis method was used in the present study to concentrate HAV, and the efficiency of this method was compared with the MNP capture assay.
3.2. Materials and methods

3.2.1. Quantification of HAV RNA via real-time RT-PCR

HAV strain HM175/24A was propagated in fetal rhesus monkey kidney cell line (FRhk-4) and was titrated by plaque assay as described in Chapter 2. HAV RNA was extracted from 200 µL of viral stock using the QIAamp MinElute Virus Spin Kit (Cat. No. 57704, Qiagen, Toronto, ON, Canada) following the manufacturer’s instructions. Briefly, 200 µL of virus were mixed with 25 µL of Qiagen protease and 200 µL of buffer AL in the Kit. The mixture was incubated at 56°C for 15 min, followed by the addition of 250 µL of ethanol (100%) and incubation at room temperature for 5 min. The sample was loaded onto the QIAamp MinElute column and centrifuged at 6,000 × g for 1 min using a Beckman Coulter Allegra 21 centrifuge with an F2402H rotor (Mississauga, ON, Canada). After washing the column sequentially with 500 µL of buffer AW1, AW2, and ethanol (100%) by centrifugation at 6,000 × g for 1 min, the column was dried by centrifugation at 20,000 × g for 3 min. Forty µL of RNase-free water (Cat. No. AM9937, Life Technologies, Burlington, ON, Canada) were used to elute RNA from the column. Ten µL of the extracted RNA were mixed with 2 µL of 10× RT buffer, 2 µL of RT random primers, 0.8 µL of 25× dNTP mix, 1 µL of multiscribe reverse transcriptase, 1 µL of RNase inhibitor, and 3.2 µL of RNase-free water from the High-capacity cDNA Reverse Transcription Kit (Cat. No. 4374966, Life Technologies, Burlington, ON, Canada) in a 20-µL reaction system. The reagents were mixed on ice. The mixture was incubated using an Applied Biosystems GeneAmp PCR system 9700 (Life Technologies, Burlington, ON, Canada) for 10 min at 25°C, 120 min at 37°C, and 5 min at 85°C, and then at 4°C until PCR amplification.
Real-time PCR was performed with a ViiA 7 system (Applied Biosystems) using primers and probe: 5’ATAGGGTAACACGCGGATAT3’ (forward), 5’CTCAATGCATCCACTGGA TGAG3’ (reverse) and 5’FAM-CCATTCAACGCGAGG-MGB3’ (Taqman probe) (Gardner et al., 2003). The PCR reaction was performed in a 20-µL mixture consisting of 10 µL of 2 × Taqman Fast Advanced Master Mix (Cat. No. 4444557, Life Technologies, Burlington, ON, Canada), 700 nM of each primer, 250 nM of probe, and 4 µL of cDNA. Thermal cycling conditions included 50°C for 2 min and 95°C for 20 s, followed by 40 cycles of 95°C for 1 s and 60°C for 20 s. Fluorescence was measured at 60°C in each cycle during the reaction. A standard curve was constructed using 10-fold serial dilutions of cDNA. The amplification efficiency was calculated using the formula E=10^{-\text{slope}}-1 (Klein et al., 1999).

3.2.2. Comparison of different RNA extraction methods

The efficiency of three methods was compared to extract RNA from concentrated HAV particles using MNPs. The three methods were: HCl and Kit method, Kit method, and Heating method. RNA from each method was quantified using real-time RT-PCR as described previously. The detailed procedures for each method are as follows:

**HCl and Kit method:** Fifty µL of PMNPs or NMNPs were inoculated into 50 mL of glycine buffer (0.05 M glycine, 0.14 M NaCl, 0.2% (v/v) Tween 20, pH 9.0) containing 8.3 \times 10^4 PFU of HAV particles. The mixture was rotated at 10 rpm at room temperature for 20 min, followed by a magnetic capture for 30 min. The supernatant was discarded and 200 µL of HCl (pH = 1) were added to release viral particles from the MNPs by vortexing for 15 min. After magnetic capture for 5 min, the viral suspension was transferred to a new 1.5 mL Eppendorf
centrifuge tube and neutralized with NaOH solution (1 M). Two hundred µL of the suspension were used for RNA extraction using the QIAamp MinElute Virus Spin Kit as described previously. RNA was eluted from the column using 40 µL of RNase-free water. The extracted RNA was reverse transcribed to cDNA as described previously.

**Kit method:** The procedure was similar to the HCl and Kit method except that after the concentration of HAV by magnetic capture, the MNPs were resuspended in 200 µL of PBS (pH 7.4, Cat. No. 10010031, Life Technologies, Burlington, ON, Canada) and vortexed for 5 min to separate the MNPs. Two hundred µL of buffer AL (with carrier RNA) and 25 µL of protease in the QIAamp MinElute Virus Spin Kit were added to the tube, mixed well, and incubated at 56°C for 15 min to lyse the virus capsid. The MNPs were separated from the lysate by magnetic capture and the lysis buffer containing the released HAV RNA was transferred to a new 1.5 mL Eppendorf centrifuge tube. Two hundred and fifty µL of ethanol (100%) were added to the tube, vortexed for 15 s, and kept at room temperature for 5 min. Then the sample was loaded onto the MinElute Spin column and the fluid was passed through the column by centrifugation at 6,000 × g for 1 min. The column was sequently washed with 500 µL of buffer AW1, AW2, and ethanol (100%) by centrifugation at 6,000 × g for 1 min, followed with drying by centrifugation at 20,000 × g for 3 min. Forty µL of RNase-free water (Cat. No. AM9937, Life Technologies, Burlington, ON, Canada) were used to elute viral RNA. The extracted RNA was reverse transcribed to cDNA as described previously.

**Heating method:** The procedure was also similar to the HCl and Kit method except that after the concentration of HAV by magnetic capture, the MNPs were resuspended in 40 µL of
RNase-free water. The mixture was sonicated at 40 kHz in a water bath sonicator (FS20, Fisher Scientific, Ottawa, ON, Canada) for 1 min to separate MNPs, and then heated at 95°C for 5 min to lyse the virus capsid. The released viral RNA was separated from MNPs by a magnet and 10 µL of the RNA was reverse transcribed to cDNA as described previously.

3.2.3. Separation and concentration of HAV from different foods artificially contaminated with HAV

Green onions and strawberries were purchased from a local supermarket, stored at 4°C, and processed within 24 h. Around 15 g of green onions (cut into ~3 cm pieces) or 50 g of strawberries (2–3 pieces) were weighed in a clean sterile petri dish and allowed to dry in a biosafety cabinet. One hundred µL of HAV dilution prepared in PBS (pH 7.4, Cat. No. 10010031, Life Technologies, Burlington, ON, Canada) containing $8.3 \times 10^5$, $8.3 \times 10^3$, $8.3 \times 10^1$, or $8.3 \times 10^0$ PFU viral particles were pipetted evenly onto the surface of green onions or strawberries with 3 µL inoculated at each spot. Each dilution was inoculated onto three independent samples and one uninoculated sample was used as a negative control. After the inoculum was dry, the green onions or strawberries were transferred to a Whirl-pack™ sterile filter bag (Cat. No. 0181269, Fisher Scientific, Ottawa, ON, Canada). Fifty mL of glycine buffer (0.05 M glycine, 0.14 M NaCl, 0.2% (v/v) Tween 20, pH 9.0) were added to the bag. The inoculated viral particles were eluted from the food surface by gently agitating the stomacher bag for 15 min. Then, the eluate was transferred to a sterile centrifuge tube (50 mL) and centrifuged at $3,500 \times g$ for 15 min to be clarified using a Beckman Coulter Allegra 21 centrifuge with an S4180 rotor (Mississauga, ON, Canada). The supernatant was transferred to a new 50 mL centrifuge tube for further analysis. In order to understand whether the
centrifugation step was necessary or not for clarification of the eluate from fresh produce, the centrifugation step was omitted in some experiments, the results of which were compared with the assay involving centrifugation.

New Zealand greenshell mussels (Talley’s Group Ltd.) were purchased from a local grocery store and kept at -80 °C until use. Ten pieces of mussel were aseptically shucked, and the whole tissue of each mussel was removed from the shell and pooled together, which was then finely chopped using sterile scissors (Greening & Hewitt, 2008). The tissue was divided into 5 g portions in a sterile petri dish. One hundred µL of HAV dilutions (8.3 × 10^5, 8.3 × 10^3, 8.3 × 10^1, or 8.3 × 10^0 PFU/inoculum) were inoculated evenly onto the tissue and was then incubated for 1 h at room temperature. One portion of mussel tissue without viral inoculum was used as a negative control. The tissue was then transferred into a Whirl-pack™ sterile filter bag. Fifty mL of glycine buffer (0.05 M glycine, 0.14 M NaCl, 0.2% (v/v) Tween 20, pH 9.0) were added to the bag and then sonicated for 5 min at 40 kHz in a water bath sonicator (FS20, Fisher Scientific, Ottawa, ON, Canada) (Morales-Rayas et al., 2009) and shaken gently at 10 rpm for 15 min. The elution fluid was transferred to a sterile 50 mL centrifuge tube and centrifuged at 4,100 × g for 20 min. The supernatant was transferred to a new 50 mL centrifuge tube for further analysis.

Pasteurized partly skimmed milk (2%, Neilson, Canada) was purchased from a local supermarket and stored at 4°C. Forty mL of the milk (in 50 mL centrifuge tube) were inoculated with 100 µL of HAV dilutions prepared in PBS (pH 7.4). The concentration of HAV in the inoculum was 8.3 × 10^6, 8.3 × 10^4, 8.3 × 10^2, or 8.3 × 10^1 PFU/mL, respectively.
The artificially contaminated milk was mixed well and then left at room temperature for 1 h. One tube of milk without HAV was used as a negative control. Four methods were employed to treat the milk before concentrating viral particles: (1) no treatment, (2) milk was centrifuged at 4,100 × g for 20 min at 4°C to be separated into three layers. The upper layer of cream and the lower layer of milk somatic cells were removed. The middle layer of skim milk was transferred to a new centrifuge tube, (3) two mL of HCl (1 N) were added to the milk to adjust the pH to 4.6, which resulted in coagulation of milk protein. The protein was removed by centrifugation at 4,100 × g for 20 min at 4°C. The supernatant was transferred to a new centrifuge tube, (4) six µL of chymosin (CHY-MAX® Extra, Cat. No. 73863, Chr. Hansen, Milwaukee, WI, USA) were added to 40 mL of milk and clotting was allowed to take place at 37°C for 20 min. Milk was then centrifuged at 4,100 × g for 20 min at 4°C again to precipitate protein. The supernatant was transferred to a new centrifuge tube for further analysis.

The virus in each processed sample was concentrated using MNPs as described in Section 3.2.2. For PMNPs, viral RNA was extracted using the Kit method. For NMNPs, the HCl and Kit method was used for viral RNA extraction. For each milk sample, before conducting RNA extraction from the concentrated virus, the MNPs were washed with 1 mL of glycine buffer once to remove the residual milk components.

3.2.4. Comparison of HAV recovery from inoculated and spiked sample using PMNPs

HAV inoculum (8.3 × 10^5 PFU) was also directly spiked into glycine buffer that had been used to wash green onions, strawberries, and mussels not inoculated with virus. The spiked virus in the elution buffer was then concentrated with PMNPs as described previously. The
recovered virus was quantified using real-time RT-PCR. The percentage of virus recovered from spiked elution buffer and inoculated food was compared. This process was used to estimate whether the recovery of HAV from different foods was decided by the elution efficiency of glycine buffer (0.05 M glycine, 0.14 M NaCl, 0.2% (v/v) Tween 20, pH 9.0) or the capacity of MNPs.

3.2.5. Concentrating HAV by PEG dialysis method

Fifty mL of glycine buffer (0.05 M glycine, 0.14 M NaCl, 0.2% (v/v) Tween 20, pH 9.0) inoculated with HAV (8.3 × 10^5 PFU) were added to a standard regenerated cellulose Spectra/Pro 3 dialysis tube (Cat. No. 132724, Spectrum Laboratories, Rancho Dominguez, California, USA) with a MWCO of 3.5 kDa. PEG with an average molecular weight of 20 kDa was used as the stressing polymer. Different concentrations (10%, 20%, or 40%, w/v) of PEG water solutions were prepared. The dialysis tube was put into the PEG reservoir (500 mL) at room temperature until the volume of the virus suspension was reduced to less than 1 mL. The optimal PEG concentration for concentrating HAV suspension was selected and used in the following experiments.

For the processing of green onions, strawberries and mussels, HAV was inoculated and released from food samples as described previously. Then, ~50 mL of clarified eluate of HAV were concentrated to less than 1 mL using the PEG dialysis method. For milk, the contaminated 40 mL of milk sample were treated with chymosin to remove milk protein and the sample was then concentrated using the PEG dialysis. Virus present in 200 µL of the concentrated suspension was quantified using real-time RT-PCR as described in previous
sections. The sensitivity of the method was tested by concentrating three different inoculum levels \((8.3 \times 10^3, 8.3 \times 10^1, \text{or} \ 8.3 \times 10^0 \text{ PFU})\) of viral particles from different foods. Each inoculum level was tested in three independent experiments. For each food, one sample without virus inoculum was used as a negative control.

3.2.6. Statistical analysis

One-way analysis of variance (ANOVA) was used to determine statistical significance. Statistical analyses were carried out using IBM SPSS Statistics (version 21; IBM Corporation, New York, USA) with \(P\)-value < 0.05 indicated a significant difference.

3.3. Results

3.3.1. Quantification of HAV via real-time RT-PCR

The titer of the propagated HAV HM175/24A determined by plaque assay was \(8.3 \times 10^6 \text{ PFU/mL}\). A standard curve of real-time RT-PCR \((y = -3.327 x + 40.238, R^2 = 0.998)\) shown in Figure 3.1 was obtained by analyzing the Ct values of 10-fold serial dilutions of cDNA. The slope demonstrated a PCR efficiency of 99.7%. The limit of detection of the real-time RT-PCR assay was 8.3 PFU/mL.
Figure 3.1. Standard curve of real-time RT-PCR made by 10-fold serial dilutions of HAV cDNA using ViiA 7 system. Each value is the mean of three independent replicates. Bars show standard deviation.

3.3.2. Comparison of different RNA extraction methods

Three different methods (HCl and Kit method, Kit method, and Heating method) were compared for extracting RNA from the viral particles concentrated from 50 mL of glycine buffer by PMNPs and NMNPs (Table 3.1). The Ct values given by real-time RT-PCR reflected the RNA extraction efficiency of different methods. The lower the Ct value, the higher the concentration of RNA in the reaction, and hence the higher the efficiency of RNA extraction method. For PMNPs, the Kit method demonstrated higher efficiency than HCl and Kit method and Heating method ($P < 0.01$). For NMNPs, the HCl and Kit method worked the best ($P < 0.01$). Therefore, the Kit method was used for studies involving PMNPs, and the HCl and Kit method was used for NMNPs in the following experiments.
Table 3.1. Comparison of different RNA extraction methods.

<table>
<thead>
<tr>
<th>RNA release method</th>
<th>Ct value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>NMNPs</td>
</tr>
<tr>
<td>HCl and Kit</td>
<td>20.8 ± 0.4</td>
</tr>
<tr>
<td>Kit</td>
<td>29.3 ± 0.4</td>
</tr>
<tr>
<td>Heating</td>
<td>ND</td>
</tr>
</tbody>
</table>

HAV concentration was $8.3 \times 10^4$ PFU/50 mL and the volume of MNPs used was 50 µL; Each value is the mean ± standard deviation (n = 3); ND means not detectable; PMNPs: protamine-coated magnetic nanoparticles; NMNPs: NH$_2$-coated magnetic nanoparticles; For each MNP, the differences of Ct values of RNA among three methods were significant ($P < 0.01$).

3.3.3. Comparison of different methods for concentrating HAV from artificially contaminated green onions, strawberries, and mussels

Different methods of concentrating HAV from artificially contaminated green onions, strawberries, and mussels were comparatively evaluated by real-time RT-PCR (Table 3.2). The sensitivity of each method was evaluated by testing four inoculum levels ($8.3 \times 10^5$, $8.3 \times 10^3$, $8.3 \times 10^1$, or $8.3 \times 10^0$ PFU) of HAV. Each inoculum level was tested in three independent samples. One sample not containing HAV was used as a negative control. HAV was not detectable in all uncontaminated samples. For the PEG dialysis method, the concentration of PEG solution was optimized for concentrating viral particles suspended in 50 mL of glycine buffer. The viral suspension ($8.3 \times 10^5$ PFU) was dialyzed against 500 mL of PEG dissolved in distilled water to obtain concentrations of 10%, 20%, or 40% (w/v) at room temperature. Results showed that the sample volume was reduced from 50 mL to less than 1 mL in about 7 h, 26 h, and 48 h, using solutions containing 40%, 20%, and 10% PEG (w/v),
respectively. Therefore, the solution with a PEG concentration of 40% (w/v) was used in future experiments.

For green onions, the sensitivities of the four concentration methods were the same \((8.3 \times 10^0\) PFU/15 g) (Table 3.2). The PEG dialysis method showed the best repeatability, because HAV was detectable in samples at each inoculum level of the virus. PMNPs tended to show more consistent positive results than NMNPs when used to separate HAV from green onions (15 g) inoculated with \(8.3 \times 10^1\) or \(8.3 \times 10^0\) PFU (Table 3.2). Furthermore, we found that there was no difference in the detection limits of HAV concentrated from the green onions rinses treated with and without the centrifugation step (Table 3.2). This indicated that centrifugation was not necessary for clarifying the eluate from green onions and also indicated that the components of green onions might not be concentrated by PMNPs or they did not inhibit the efficiency of real-time RT-PCR.

As for the HAV recovery from strawberries, PMNPs and NMNPs demonstrated a detection limit of \(8.3 \times 10^1\) PFU/50 g and \(8.3 \times 10^3\) PFU/50 g, respectively, according to the selected inoculum levels of HAV in our study. Compared with NMNPs, PMNPs produced more consistent positive results at each inoculum level of the virus (Table 3.2). The centrifugation step was essential for clarifying the eluate from strawberries, due to the fact that the detection limit of HAV in centrifugation-clarified strawberry eluate was \(8.3 \times 10^1\) PFU/50 g but HAV was not detected at all in each strawberry sample prepared without the pretreatment with centrifugation (Table 3.2). This result indicated that the components of strawberries might interact with PMNPs and they competed with HAV particles for PMNPs, resulting in low
recovery rates of HAV. For the PEG dialysis method, HAV could not be detected in any of the artificially contaminated samples. These results implied that components of strawberries might strongly inhibit the efficiency of real-time RT-PCR.

As for mussels, the three methods shared the same detection limit of $8.3 \times 10^0$ PFU/5 g (Table 3.2). The PEG dialysis method demonstrated the best consistency. More true positive results were obtained when PMNPs were used as opposed to NMNPs. Because a centrifugation step is recommended for concentrating viruses from shellfish (Casas et al., 2007; Mesquita et al., 2011; Pintó et al., 2009), this step was not evaluated in the current study.
Table 3.2. Concentration of HAV from green onions, strawberries, and mussels using different methods.

<table>
<thead>
<tr>
<th>Food sample</th>
<th>Method</th>
<th>No. of positive samples / No. of samples analyzed at inoculation level (PFU) of</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>$10^5$</td>
</tr>
<tr>
<td>Green onion</td>
<td>PMNPs with centrifugation</td>
<td>3/3</td>
</tr>
<tr>
<td></td>
<td>PMNPs without centrifugation</td>
<td>3/3</td>
</tr>
<tr>
<td></td>
<td>NMNPs with centrifugation</td>
<td>3/3</td>
</tr>
<tr>
<td></td>
<td>PEG dialysis</td>
<td>NA</td>
</tr>
<tr>
<td>Strawberry</td>
<td>PMNPs with centrifugation</td>
<td>3/3</td>
</tr>
<tr>
<td></td>
<td>PMNPs without centrifugation</td>
<td>0/3</td>
</tr>
<tr>
<td></td>
<td>NMNPs with centrifugation</td>
<td>2/3</td>
</tr>
<tr>
<td></td>
<td>PEG dialysis</td>
<td>NA</td>
</tr>
<tr>
<td>Mussel</td>
<td>PMNPs</td>
<td>3/3</td>
</tr>
<tr>
<td></td>
<td>NMNPs</td>
<td>3/3</td>
</tr>
<tr>
<td></td>
<td>PEG dialysis</td>
<td>NA</td>
</tr>
</tbody>
</table>

PMNPs: protamine-coated magnetic nanoparticles; NMNPs: NH$_2$-coated magnetic nanoparticles; NA: not analyzed; Virus was recovered on three distinct occasions and detection of viral RNA by real-time RT-PCR was carried out in duplicates. A sample was considered positive if both tests of viral RNA were positive.

3.3.4. Comparison of HAV recovery from inoculated and spiked samples using protamine-coated MNPs

The separation and concentration of viral particles from solid food samples included two steps: elution of virus from food surface and concentration of virus to a small volume. In order to understand which step determined the overall recovery efficiency of HAV, two processes called inoculation and spiking were evaluated in the current study. For inoculation, 100 µL of
HAV (8.3 × 10^6 PFU/mL) were inoculated on green onion, strawberry, and mussel sample, and then eluted with 50 mL of glycine buffer. For spiking, the glycine buffer was firstly used to wash the surface of uncontaminated foods, and then 100 µL of HAV (8.3 × 10^6 PFU/mL) were directly spiked into the rinsing solution. Both inoculated and spiked eluates were clarified with centrifugation, and virus was then concentrated by PMNPs as described previously. Results showed that there was no significant difference in HAV recovery rate between the inoculated and spiked samples for green onions (P = 0.183), strawberries (P = 0.933), and mussels (P = 0.135) (Table 3.3), indicating that immersing food in glycine buffer (pH 9.0) and gently shaking the sample for 15 min (with extra sonication for 5 min for mussels) was efficient in breaking the binding force of HAV on food surface. Therefore, the recovery of virus from the inoculated samples was dependent on the virus-binding capacity of PMNPs.
Table 3.3. Recovery rate of HAV from inoculated and spiked samples using PMNPs.

<table>
<thead>
<tr>
<th>Food</th>
<th>Inoculated sample</th>
<th>Spiked sample</th>
</tr>
</thead>
<tbody>
<tr>
<td>Green onion</td>
<td>12.0 ± 2.4</td>
<td>10.4 ± 1.4</td>
</tr>
<tr>
<td>Strawberry</td>
<td>11.7 ± 0.7</td>
<td>11.7 ± 0.8</td>
</tr>
<tr>
<td>Mussel</td>
<td>1.4 ± 0.4</td>
<td>2 ± 0.7</td>
</tr>
</tbody>
</table>

§PMNPs: protamine-coated magnetic nanoparticles; Recovery rate = total viral particles captured / total viral particles input before separation; The inoculum level of HAV was 8.3 × 10⁵ PFU; Inoculated sample: HAV was inoculated on food, eluted with 50 mL of glycine buffer, and then concentrated to a small volume; Spiked sample: each food was washed with glycine buffer firstly, and then HAV was inoculated into the eluate, followed with the concentration of HAV using PMNPs; Each value is the mean ± standard deviation (n=3); For each food, the difference of recovery rate of HAV between inoculated and spiked sample was not significant, with P values of 0.183, 0.933, and 0.135 for green onion, strawberry, and mussel, respectively.

3.3.5. Comparison of different methods for concentrating HAV from artificially contaminated milk

Casein comprises around 80% of milk protein (DeMan, 1999). The initial pH of milk is around 6.6. At this pH, casein is negatively charged, because the pI of casein is 4.6 (DeMan, 1999). Thus, casein could be attracted by positively charged MNPs and the presence of casein in milk probably interferes with the electrostatic attraction between HAV and MNPs. Two different approaches were applied in the current study to remove casein from milk: chymosin precipitation and HCl precipitation. In addition to these two treatments, milk was also simply centrifuged to remove fat and somatic cells before virus recovery. The results showed that the combination of chymosin precipitation and PMNP capture had greater repeatability than other
methods (Table 3.4). However, these treatments did not improve the detection limit of HAV as the value for untreated milk was also 8.3 PFU/40 mL. Milk components could not penetrate the dialysis membrane, so the PEG dialysis method was only applied to milk, from which casein was precipitated by chymosin treatment. The PEG dialysis method demonstrated a detection limit of 8.3 PFU/40 mL of HAV, which was comparable with PMNP treatment (Table 3.4).

### Table 3.4. Detection of HAV from milk pre-treated with different methods.

<table>
<thead>
<tr>
<th>Treatment before virus concentration</th>
<th>Virus concentration method</th>
<th>No. of positive samples / No. of tested samples at inoculation level (PFU) of $10^5$</th>
<th>$10^3$</th>
<th>$10^1$</th>
<th>$10^0$</th>
</tr>
</thead>
<tbody>
<tr>
<td>No treatment</td>
<td>PMNPs</td>
<td>3/3</td>
<td>3/3</td>
<td>1/3</td>
<td>1/3</td>
</tr>
<tr>
<td></td>
<td>NMNPs</td>
<td>3/3</td>
<td>3/3</td>
<td>1/3</td>
<td>0/3</td>
</tr>
<tr>
<td>Centrifugation</td>
<td>PMNPs</td>
<td>3/3</td>
<td>3/3</td>
<td>1/3</td>
<td>0/3</td>
</tr>
<tr>
<td></td>
<td>NMNPs</td>
<td>3/3</td>
<td>3/3</td>
<td>0/3</td>
<td>0/3</td>
</tr>
<tr>
<td>HCl precipitation</td>
<td>PMNPs</td>
<td>3/3</td>
<td>3/3</td>
<td>1/3</td>
<td>0/3</td>
</tr>
<tr>
<td></td>
<td>NMNPs</td>
<td>3/3</td>
<td>3/3</td>
<td>1/3</td>
<td>0/3</td>
</tr>
<tr>
<td>Chymosin precipitation</td>
<td>PMNPs</td>
<td>3/3</td>
<td>3/3</td>
<td>2/3</td>
<td>1/3</td>
</tr>
<tr>
<td></td>
<td>NMNPs</td>
<td>3/3</td>
<td>3/3</td>
<td>1/3</td>
<td>0/3</td>
</tr>
<tr>
<td></td>
<td>PEG dialysis</td>
<td>NA</td>
<td>3/3</td>
<td>1/3</td>
<td>1/3</td>
</tr>
</tbody>
</table>

$^5$PMNPs: protamine-coated magnetic nanoparticles; NMNPs: NH$_2$-coated magnetic nanoparticles; NA: not analyzed; The inoculum level of HAV was $8.3 \times 10^5$, $8.3 \times 10^3$, $8.3 \times 10^1$, or $8.3 \times 10^0$ PFU/40 mL of milk; Virus was recovered on three distinct occasions and detection of viral RNA by real-time RT-PCR was carried out in duplicates. A sample was considered positive if both tests of viral RNA were positive.
3.4. Discussion

The four foods selected in the current study for investigating the efficiency of different methods for concentrating HAV have been implicated in cases of viral foodborne infections. Green onions, strawberries, and mussels have been frequently used in HAV study, but for milk, it is seldom used as a food vehicle for virus research. Different amounts (5 – 100 g) of fruits and vegetables were used in previous studies (Cheong et al., 2009; Dubois et al., 2002; Lee et al., 2012; Rzeutka et al., 2006; Sánchez et al., 2012; Summa et al., 2012). We selected 15 g of green onions and 50 g of strawberries (2 – 3 pieces) in our research, since this sample size could be conveniently submerged in 50 mL of glycine buffer in a Whirl-pack™ sterile filter bag. Covering the surface of food ensured that the viral particles on the surface were readily eluted by the rinsing buffer. The whole tissue of mussel was used for virus contamination rather than only the digestive gland, because viruses are not only found in digestive tissues (stomach, intestine, and digestive diverticula), but also in nondigestive tissues (gill, labial palp, adductor muscle, and mantle tissue) (McLeod et al., 2009). The viral particles in digestive tract lumen are readily eliminated through depuration in clean water, but the particles within the cells of digestive and nondigestive tissues were not easily depurated or inactivated. Although the load of virus in nondigestive tissues is lower than that in digestive tissues (Wang et al., 2008), the low infectious dose of virus (10 – 100 particles) makes detection of virus in nondigestive tissues important (Yezli & Otter, 2011). Milk was reported to be contaminated with HAV (Murphy et al., 1946; Zaher et al., 2008). Fat in milk can protect HAV from inactivation by heat, and the higher the fat content, the stronger the heat stability of HAV (Bidawid et al., 2000a). Thus, pasteurization (63°C for 30 min or 72°C for 15 s) cannot guarantee the complete inactivation of HAV in milk. The source of contamination might also
be from post-pasteurization contamination by infected food handlers or the breakdown of pasteurizer in the processing. Since pasteurized milk is at a high risk of contamination with enteric viruses, we chose it as a food vehicle in the current study.

Since it was extremely difficult to obtain naturally contaminated food samples, we could only use artificially contaminated food in our research. The mode of seeding virus in food should be as close as possible to those found in nature. However, none of the methods could completely mimic the real situation. For artificially contaminating fresh produce, spraying may be appropriate, however, the reproducibility of this approach cannot be guaranteed. Inoculating a viral suspension on many different spots on the food has been widely used in foodborne virus research (Coudray et al., 2013; Dubois et al., 2002; Martin-Latil et al., 2012; Sánchez et al., 2012). Bianchi et al. (2011) homogenized strawberries before artificially contaminating the sample with HAV to ensure the reproducibility of subsamples, but there was no report of viruses found inside strawberries. Another drawback of this method is that PCR inhibitors inside strawberries may be released. For artificial contamination of mussels, in order to make sure the whole mussel was contaminated with HAV, we chopped the whole tissue finely and inoculated virus evenly on the mud. In this way, we also guaranteed that the mussel tissue could be macerated to release viral particles from the tissue cell. It was reported that manually chopping the tissue was as effective as homogenization of shellfish (Greening & Hewitt, 2008).

For using slow speed centrifugation to clarify the rinse fluid from fresh produce prior to virus recovery, we found that it was essential to clarify strawberry eluate, but the centrifugation step
could be omitted for green onion samples. In a study by Guévremont et al. (2006), the rinse fluid from green onions was directly processed by a PEG precipitation protocol to concentrate HAV and NoV without clarification treatment. The methodologies used in their study demonstrated a detection limit of 1 TCID$_{50}$ of HAV per 25 g of green onions. In another study by Rzeutka et al. (2006), slow speed centrifugation was used to pretreat the eluate from soft berry fruits including raspberries and strawberries to remove fruit debris. HAV in the clarified eluate was concentrated using an ultracentrifugation protocol coupled with nested RT-PCR detection. The overall method was shown to be able to detect $10^4$ RT-PCR unit of HAV in 90 g of strawberries and $10^3$ RT-PCR unit of HAV in 60 g of raspberries. In the above-mentioned studies, the researchers did not evaluate the essentiality of clarification of eluate from green onions and strawberries. In our study, green cellular materials after elution were found in the rinse fluid of green onions but did not significantly affect the MNP capture efficiency, because the detection limit of HAV was the same (8.3 PFU per 15 g of green onions) with or without the removal of the cellular materials by slow speed centrifugation. For strawberry eluate, centrifugation was rather necessary to remove the red cellular materials found in the rinsed solution. Without clarification of the strawberry eluate, HAV at each inoculum level could not be detected. The results indicated that food matrix had a great impact on virus recovery. Pretreatment of food was of great importance for improving the sensitivity of foodborne virus detection. Different foods required different pretreatment procedures to eliminate the inhibitory effect of food components, because each food matrix had different physical characteristics and might carry different inhibitory compounds.
Two different cationic MNPs were fabricated in our study for the concentration of HAV. Results showed that PMNPs had an overall better performance than NMNPs with regards to sensitivity and repeatability. In our experiment, both MNPs were stored at 4°C after production. We found that PMNPs could be suspended in water for longer time than NMNPs during storage, indicating that the former was more stable than the latter. The stability of MNPs might affect the capturing efficiency of virus. Another explanation of better performance of PMNPs is the attraction force between PMNPs and HAV particles. As is shown in Figure 2.3, the pI of PMNPs is higher than NMNPs, making the electrostatic attraction force between PMNPs and viral particles stronger than that between NMNPs and the virus. Aside from electrostatic attraction force, hydrophobic interactions and hydrogen bonding between PMNPs and HAV capsid may contribute to the better performance of PMNPs. Cationic MNPs with different surface modification exhibited varying concentration efficiencies for the same virus, which was in good agreement with the results obtained by Uchida et al. (2007). In their research, three cationic magnetic beads, polyethylenimine (PEI)-coated, polyarylamine (PAA)-coated, and poly-L-lysine (PLL)-coated magnetic beads, were used to concentrate different viruses including HAV, HBV (hepatitis B virus), HCV (hepatitis C virus), SV-40 (simian virus 40), HSV-1 (herpes simplex 1 virus), PPV (porcine parvovirus), and adenovirus from viral suspension in cell culture medium. The PEI-coated magnetic beads showed the highest virus recovery efficiency and the beads coated with PEIs of different molecular weights showed dramatic different efficiency in capturing viruses (Uchida et al., 2007). We used protamine in our research instead of PEI, since the latter is cytotoxic and hazardous to human health (Hunter, 2006; Moghimi et al., 2005).
The use of PMNPs allowed detection of 8.3 PFU of HAV in green onions, mussels, and milk, and 83 PFU of HAV in strawberries. The limit of detection of this technique is believed to satisfy the need for diagnostic applications in a food microbiological laboratory, because HAV infectious dose is between 10 and 100 viral particles (Yezli & Otter, 2011). The detection limit of HAV in different foods obtained from our technique is comparable with or better than that reported by the following research groups. Guévremont et al. (2006) reported 1 TCID<sub>50</sub> (equivalent to 1.4 PFU) of HAV could be detected in 25 g of green onion sample with a PEG precipitation method used for virus concentration and RT-PCR for detection, which was similar to our result (8.3 PFU). Rzeutka et al. (2006) reported a detection limit of 10<sup>4</sup> RT-PCR unit of HAV in strawberry samples (90 g). This was much higher than the detection limit of 83 PFU (equivalent to 10 RT-PCR unit) obtained in our study. Butot et al. (2007) found that 1.2 TCID<sub>50</sub> (equivalent to approximately 1.7 PFU) of HAV could be detected in 15 g of strawberries using an ultrafiltration procedure coupled with real-time RT-PCR detection. This reported detection limit was about 50 times lower than our result (83 PFU). The difference might be because they used pectinase to pretreat strawberry eluate before concentration of HAV was conducted. In a previous study conducted by our research group for using cationic beads to concentrate HAV from strawberries and green onions, for the viral inoculum level of 10<sup>3</sup> and 10<sup>2</sup> PFU, not all three seeded samples for each produce were consistently detected (Morales-Rayas et al., 2010). In the current study, for the viral inoculum level of 10<sup>1</sup> PFU, all samples were positively detected with the use of PMNPs. For detection of HAV in shellfish, Kingsley & Richards (2001) used a PEG precipitation protocol to concentrate HAV from seeded oysters and clams and the concentrated HAV was detected by RT-PCR. The detection limit of HAV in oysters (3.75 g) was 1.5 PFU, and in clams (3.75 g)
was 0.15 PFU. In another study by Casas et al. (2007), 25 TCID$_{50}$ (equivalent to approximately 35 PFU) of HAV was detectable in seeded oysters (25 g) when the virus was concentrated using ultracentrifugation method and detected by real-time RT-PCR. Di Pasquale et al. (2010) reported 500 TCID$_{50}$ (approximately 700 PFU) of HAV was detectable in one gram of mussel. The detection limit of HAV in seeded mussels (5 g) in our study was 8.3 PFU, and the value was comparable with or better than these reported results. Milk was seldom detected for the presence of HAV. Hirneisen et al. (2009) used cationic magnetic beads to concentrate HAV from 250 mL of milk and found the sensitivity of the method was only $10^6$ TCID$_{50}$/mL (equivalent to approximately $10^6$ PFU/mL).

For the virus inoculum level of $8.3 \times 10^5$, $8.3 \times 10^3$, $8.3 \times 10^1$, or $8.3 \times 10^0$ PFU per food sample, the recovery rate of HAV obtained from 15 g of green onions by PMNPs (with centrifugation involved) was 3%, 12%, 9.2% and 32.3%, respectively; the recovery rate of HAV from 50 g of strawberries was 0.9%, 4.1%, 10.5% and 0, respectively; the recovery rate of HAV from 5 g of mussel was 0.3%, 1.5%, 2.7% and 18.2%, respectively; the recovery rate of HAV from 40 mL of milk (no pre-treatment) was 0.1%, 0.2%, 0.7% and 4.9%, respectively. With the increase of virus inoculum level, the recovery rate of HAV decreased in each food, implying that food components might compete with viral particles for PMNPs and the concentration of PMNPs (50 µL) was not enough for capturing high contamination levels of virus in food. However, because the natural contamination level of virus in food is usually low, the purpose of foodborne virus detection is trying to test virus at the lower contamination levels, thus, 50 µL of PMNPs are still appropriate for performing the experiment. The same tendency was also reported by Fumian et al. (2009) for recovering different inoculum levels of
NoVs from lettuce and cheese using a negatively charged membrane filtration protocol. Their results showed that with the decrease of NoV inoculum levels in food samples, the recovery rate of NoVs in lettuce increased from 5.2% to 72.3% and in cheese increased from 6% to 56.3%. Martin-latil et al. (2012) found that when a combined protocol of PEG precipitation and ultracentrifugation was used to concentrate HAV from semidried tomatoes, 50% and 13% of HAV particles were recovered for the inoculum level of 1 PFU and 1000 PFU, respectively. In a previous study, 1.7% – 2.6% of HAV was recovered from mussels (1 g) seeded with different levels of HAV between $5 \times 10^7$ and $5 \times 10^1$ TCID$_{50}$ using a proteinase K treatment method (Di Pasquale, et al., 2010). The recovery efficiency of HAV in that study was comparable with our results. Only limited published data could be used for comparing the recovery rates of HAV in green onions, strawberries, and milk. Sun et al. (2012) reported 31% of HAV could be recovered from green onions but the researchers only tested two inoculum levels of HAV in green onion samples: $10^3$ and $10^5$ PFU. Deboosere et al. (2012) reported 24.1% of HAV could be recovered from strawberries but the researchers also only tested one inoculum level ($10^6$ PFU) of the virus. In a study by Hirneisen et al. (2009), between 33.8% and 49% of HAV could be recovered from milk for the inoculum level of $10^6$ or $10^7$ TCID$_{50}$/mL.

The PMNP capture method developed in our study took approximately 50 min to complete the concentration of virus from food samples. The whole procedure took approximately 4.5 h to perform, including sample pretreatment, virus concentration, RNA extraction and real-time RT-PCR amplification. Compared with the existing methods, the developed technique is easy-
to-perform without the need of any special infrastructure, is suitable for the detection of viruses from various foods, and can be used in laboratories where resources are limited.

The PEG dialysis method used in the current study performed well for green onions, mussels, and milk, making 8.3 PFU of HAV detectable, but was incapable of making any level of HAV detectable in strawberries by real-time RT-PCR. The problem associated with strawberries might be caused by the inhibition effect of pectin on real-time RT-PCR. The problem might be solved by the use of pectinase to treat strawberry eluate (Butot et al., 2007). The PEG dialysis method used in our study took 8 h to concentrate food eluate with a large volume to a sample with small volume, which was much longer than PMNP method (50 min). Even though the PEG dialysis method was time-consuming, it provided higher virus recovery rates than the PMNP method in concentrating HAV from green onions. When used for capturing HAV from green onions with a contamination level of 8.3 PFU/15 g, the PEG dialysis method and the PMNP method (with centrifugation) demonstrated HAV recovery rates of 98.5% and 32.3%, respectively. So, the PEG dialysis method seems to be more suitable to process eluate of food with smooth and solid surface. We used a static PEG dialysis method in the current study. The PEG solution was in a beaker and was not circulated. In order to significantly reduce the processing time of the PEG dialysis method, a dynamic dialysis process with a circulation apparatus of PEG could be designed in future. Compared with the currently used virus concentration methods, this method is also easy-to-perform and can be used anywhere without the need of special infrastructure.
3.5. Conclusions

Different methods were used in the present study to concentrate HAV from green onions, strawberries, mussels, and milk. The efficiency of each method was evaluated by two-step real-time RT-PCR. PMNPs and NMNPs performed differently in capturing HAV from the same food matrix, with PMNPs providing better sensitivity and repeatability. The PEG dialysis method performed well for concentrating HAV from green onions, but HAV could not be detected from strawberry concentrate by this method. Glycine buffer (pH 9.0) containing 0.05 M glycine, 0.14 M NaCl, and 0.2% Tween 20 (v/v) worked efficiently in recovering HAV particles from foods. After eluting HAV from strawberries, centrifuging the eluate to remove food debris was essential to ensure high sensitivity of the subsequent HAV detection. However this centrifugation step was not necessary for handling HAV-contaminated green onions. For concentrating HAV from milk, no pre-treatment of milk was needed.
Chapter 4: DETECTION OF FOODBORNE HEPATITIS A VIRUS USING A BIOLUMINESCENT REAL-TIME REVERSE TRANSCRIPTASE LOOP-MEDIATED ISOTHERMAL AMPLIFICATION TECHNOLOGY

4.1. Introduction

Recently, foodborne outbreaks of HAV have tended to increase because of increasing numbers of international travellers, mass global migration, and the fast growth global food trade. In 2013, HAV outbreaks occurred in Italy due to the consumption of contaminated frozen berries imported from Egypt and Morocco, causing over 800 people to become sick in Italy ([http://barfblog.com/tags/europe/](http://barfblog.com/tags/europe/)). Sixty-six infected individuals in Denmark, 17 in Sweden, 13 in Finland, and 7 in Norway were confirmed due to consumption of the frozen berries with the same origin (Nordic outbreak investigation team, 2013). Fifteen cases in Germany, the Netherlands, and Poland were laboratory-confirmed to be infected with HAV due to travel to Italy. In the meantime, there were 3 reported cases in Ireland, and the sequences of HAV isolated from three Irish cases were found to be identical to the Italian outbreaks, however, the source of these three cases was not clear ([http://www.efsa.europa.eu/en/supporting/pub/459e.htm](http://www.efsa.europa.eu/en/supporting/pub/459e.htm)). In 2013, 165 people from 10 states in the USA succumbed to HAV infections from the consumption of pomegranate seeds (Collier et al., 2014). These foodborne outbreaks have resulted in large economic loss to the society due to worker absenteeism caused by the disease, costs of health care, and costs of food recall and disposal, etc. The worldwide increase in the occurrence of foodborne HAV outbreaks has heightened interest in the development of
methods for fast identification of food contaminated with HAV, which is essential for implementing intervention strategies to prevent and reduce illnesses caused by the virus.

The contamination level of HAV in food is very low and viral particles cannot be enriched by cell culture before detection (Boxman et al., 2012; Felix-Valenzuela et al., 2012). As a consequence, the detection methods must be sensitive enough to be capable of quantifying this trace number of the viral particles. So far, real-time RT-PCR is the molecular biology technique most widely used to detect viruses in food. However, PCR requires thermo-cycling to denature dsDNA prior to and during amplification, imposing specific equipment requirements. PCR is also susceptible to inhibition by components found in some food samples, such as Ca^{2+} (Bickley et al., 1996), NaCl, KCl, phenol (Davalieva & Efremov, 2010), polysaccharides (Monteiro et al., 1997), collagen (Kim et al., 2000), proteinases (Bickley et al., 1996), and glycogen (Atmar et al., 1993), resulting in false-negative results.

Loop-mediated isothermal amplification (LAMP) technology is a molecular biology technique, in which a DNA polymerase with a strand displacement activity is employed (Notomi et al., 2000). The amplification of DNA is conducted under a constant temperature. The use of four primers recognizing six distinct regions of the target makes this method highly specific (Notomi et al., 2000). Large amounts of DNA are produced during amplification, leading to liberation of enormous concentration of a side product, inorganic pyrophosphate ions (PPi). Each time, when a nucleotide base is added during the polymerization reaction, a molecule of PPi is released. The amount of PPi produced is proportional to the amount of polynucleotide synthesized, and hence the concentration of starting template. The synthesized
PPI can be converted to ATP by the enzyme ATP sulfurylase using adenosine 5’-phosphosulfate (APS) as the substrate. The ATP generated is simultaneously used by firefly luciferase to oxidize its substrate luciferin to emit light (Figure 4.1). Based on this mechanism, the dynamic changes of PPI, and hence, the DNA amplification can be monitored by testing the light output in a real-time mode. During DNA amplification, with the increase of PPI liberated, the light signal increases rapidly at first, and later the PPI molecules accumulated in the reaction inhibit the light output, thus, the level of light output increases to a peak and the time at which this peak is detected reflects the concentration of the initial template (Gandelman et al., 2010; Kiddle et al., 2012). The combination of bioluminescence assay in real-time and LAMP reaction is recently described as LAMP-BART technique (Gandelman et al., 2010). This luminescence-based assay is more cost-effective than a fluorescence-based assay, for example, real-time PCR, and it is more tolerant to substances that typically inhibit PCR (Kiddle et al., 2012). In this study, RT-LAMP-BART was investigated in the detection of HAV concentrated from green onions, strawberries, mussels, and milk. The efficiency of this assay was compared with that of real-time RT-PCR.

Figure 4.1. Chemical mechanism of LAMP-BART assay (Gandelman et al., 2010).
4.2. Materials and methods

4.2.1. HAV propagation and RNA extraction

HAV strain HM175/24A was propagated in fetal rhesus monkey kidney cell line (FRhk-4) and was titrated by plaque assay as described in Chapter 2. HAV RNA was extracted from 200 µL of viral stock using the QIAamp MinElute Virus Spin Kit (Cat. No. 57704, Qiagen, Toronto, ON, Canada) following the manufacturer’s instructions. Briefly, 200 µL of virus were mixed with 25 µL of Qiagen protease and 200 µL of buffer AL in the Kit. The mixture was incubated at 56°C for 15 min, followed by the addition of 250 µL of ethanol (100%) and incubation at room temperature for 5 min. The sample was loaded onto the QIAamp MinElute column and centrifuged at 6,000 × g for 1 min using a Beckman Coulter Allegra 21 centrifuge with an F2402H rotor (Mississauga, ON, Canada). After washing the column sequentially with 500 µL of buffer AW1, AW2, and ethanol (100%) by centrifugation at 6,000 × g for 1 min, the column was dried by centrifugation at 20,000 × g for 3 min. Forty µL of RNase-free water (Cat. No. AM9937, Life Technologies, Burlington, ON, Canada) were used to elute RNA from the column. The RNA was stored at -80°C until analysis.

4.2.2. RT-LAMP of HAV

The primers used for the RT-LAMP assay of HAV are shown in Table 4.1. The RT-LAMP assay was carried out to detect HAV using the Loopamp RNA Amplification Kit (Cat. No. LMP244, Eiken Chemical Co. Ltd., Tokyo, Japan) according to the manufacturer’s instructions. Briefly, a 25 µL-reaction was composed of 12.5 µL of 2 × reaction mixture, 1 µL of enzyme mixture containing 16 U Bst DNA polymerase and 2 U avian myeloblastosis virus (AMV) reverse transcriptase, 0.8 µM of each of the FIP031 and FIP primers, 1.6 µM of BIP
primer, 0.8 μM of each of the FLOOP and BLOOP primers, 0.2 μM of each of the F3 and B3 primer, and 5 μL of extracted RNA. The reaction mixture was incubated using an Applied Biosystems GeneAmp PCR system 9700 (Life Technologies, Burlington, ON, Canada) at 62.5°C for 60 min, 80°C for 5 min, and 4°C until further analysis (Yoneyama et al., 2007). One reaction without RNA template was used as a negative control.

The RT-LAMP product was analyzed by agarose gel electrophoresis. To perform the agarose gel electrophoresis, a slab of 1% (w/v) agarose gel was prepared by melting 0.5 g of agarose powder (Cat. No. 161-3101, Bio-Rad Laboratories Ltd., Mississauga, Canada) in 50 mL of 1 × TAE buffer (Cat. No. 161-0743, Bio-Rad Laboratories Ltd., Mississauga, Canada). The mixture was boiled in a microwave until a clear solution was observed. The solution was cooled to 40 – 45°C, and then 0.5 μL of ethidium bromide (10 mg/mL, Cat. No. E1510, Sigma-Aldrich, Oakville, ON, Canada) was added to the solution. After that, the solution was poured into a 10 cm tray and solidified at room temperature. The gel was placed in a gel electrophoresis submarine cell containing 250 mL of 1 × TAE buffer. Three μL of RT-LAMP product were mixed with 1 μL of 6 × gel loading dye (Cat. No. FERR0611, Fisher Scientific, Ottawa, ON, Canada) and 2 μL of 1 × TAE buffer. The mixture was loaded in each well, and then subjected to electrophoresis at 75 V for 50 min. The bands were visualized under an UV transilluminator (Bio-Rad Laboratories Ltd., Mississauga, Canada).

4.2.3. One-step RT-LAMP-BART assay

For preparation of the reagents for RT-LAMP-BART assay, briefly, 187.5 μL of 2 × Lumopol buffer (Lumora Ltd., Ely, Cambridgeshire, United Kingdom) were used to reconstitute the
lyophilized RT-LAMP-BART-master (Lumora Ltd., Ely, Cambridgeshire, United Kingdom). Foam should be avoided in reconstituting the RT-LAMP-BART-master. Each reagent was kept on ice during the operation. All primers were mixed together using RNase-free water (Cat. No. AM9937, Life Technologies, Burlington, ON, Canada). The concentration of each primer in the primer mix was: 2.1 µM of each of the FIP031 and FIP primers, 4.3 µM of BIP primer, 2.1 µM of each of the FLOOP and BLOOP primers, and 0.5 µM of each of the F3 and B3 primers. Then, equal volumes of the reconstituted RT-LAMP-BART-master were mixed together with primer mix. The remaining reconstituted RT-LAMP-BART-master was stored at -150°C for later use. Fifteen µL of the mixture were loaded to wells of a 96-well plate (Cat. No. 14-230-232, Fisher Scientific, Ottawa, ON, Canada), 5 µL of RNA template were added to each well, and then the reaction mixture was covered with 20 – 30 µL of molecular grade mineral oil (Lumora Ltd., Ely, Cambridgeshire, United Kingdom) to prevent evaporation. In a 20 µL-reaction of one-step RT-LAMP-BART (RNA-LAMP-BART), the final concentration of primers was: 0.8 µM of each of the FIP031 and FIP primers, 1.6 µM of BIP primer, 0.8 µM of each of the FLOOP and BLOOP primers, and 0.2 µM of each of the F3 and B3 primers. The effect of different concentrations of MgSO₄ ranging from 2 mM to 4 mM on the RNA-LAMP-BART reaction efficiency was tested. The reaction plate was sealed with an adhesive film (Cat. No. 4360954, Life Technologies, Burlington, ON, Canada) and then the reaction mixture was incubated at 55°C with luminescence tested at 1 min intervals using a Bison system (Figure 4.2; Lumora Ltd., Cambridgeshire, United Kingdom) for 100 min. A standard curve was constructed by analyzing the time-to-peak of the 10-fold serial dilutions of HAV RNA. The efficiency of the reaction using primers purified with HPLC (high-performance liquid chromatography) and cartridge technique was compared. HPLC-purified primers were
synthesized by Life Technologies Corporation (Burlington, ON, Canada) and cartridge-purified primers were obtained from Laboratory Services at the University of Guelph (Guelph, ON, Canada).

4.2.4. Two-step RT-LAMP-BART assay

HAV RNA was reverse transcribed to cDNA using the High-capacity cDNA Reverse Transcription Kit (Cat. No. 4374966, Life Technologies, Burlington, ON, Canada) in accordance with the manufacturer’s instructions. Briefly, 10 µL of the extracted RNA were mixed with 2 µL of 10× RT buffer, 2 µL of RT random primers, 0.8 µL of 25× dNTP mix, 1 µL of multiscribe reverse transcriptase, 1 µL of RNase inhibitor, and 3.2 µL of RNase-free water from the kit in a 20-µL reaction. The reagents were mixed on ice. The mixture was incubated using an Applied Biosystems GeneAmp PCR system 9700 (Life Technologies, Burlington, ON, Canada) for 10 min at 25°C, 120 min at 37°C, and 5 min at 85°C, and then at 4°C until further analysis.

The preparation for the reaction of two-step RT-LAMP-BART (cDNA-LAMP-BART) was almost the same as the RNA-LAMP-BART assay except that the LAMP-BART-master (Lumora Ltd., Ely, Cambridgeshire, United Kingdom) was used, 4 µL of target cDNA were tested, and the mixture was incubated at 62°C for 100 min with luminescence tested at 1 min intervals with the Bison system (Lumora Ltd., Ely, Cambridgeshire, United Kingdom) (Figure 4.2). The effect of the concentration of MgSO₄ ranging from 0 mM to 4 mM on cDNA-LAMP-BART reaction was tested. A standard curve was constructed by analyzing the time-to-peak of 10-fold serial dilutions of HAV cDNA.
Figure 4.2. Bison system (Lumora Ltd.) for LAMP-BART assays.
Table 4.1. Details of primers used for HAV assays by real-time RT-PCR and RT-LAMP-BART§.

<table>
<thead>
<tr>
<th>Assay</th>
<th>Primer name</th>
<th>Primer sequence (5’-3’)</th>
<th>Primer position</th>
<th>Polarity</th>
<th>Reference</th>
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<tr>
<td>Real-time</td>
<td>Forward</td>
<td>ATAGGGTAAACAGCGGCGGATAT</td>
<td>448-469</td>
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<td>RT-PCR</td>
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<td>CTCAATGCACTCCACTGGATGAG</td>
<td>516-537</td>
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<td>(Gardner et al., 2003)</td>
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<td></td>
<td>Probe</td>
<td>FAM-CCATTCAACCGCCGGAG-MGB</td>
<td>492-508</td>
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<td>RT-LAMP-BART</td>
<td>F3</td>
<td>GCATGGAGCTGTAGGAGTCT</td>
<td>293-312</td>
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<td>(Yoneyama et al., 2007)</td>
</tr>
<tr>
<td></td>
<td>B3</td>
<td>CACTCAATGCACTCCACTGGA</td>
<td>520-539</td>
<td>-</td>
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<td></td>
<td>F1C</td>
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<td>385-406</td>
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<td></td>
<td>F2</td>
<td>TGTTGGGAACGTCACTTTG</td>
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<td></td>
<td>FIP031</td>
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<td></td>
<td>BIP</td>
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<td>444-464</td>
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<td>(Yoneyama et al., 2007)</td>
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<td></td>
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<td>444-464</td>
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</tbody>
</table>

§FIP and FIP031 primers consisted of F1C plus F2 and BIP primer consisted of B1C plus B2. The different bases between primer FIP and FIP031 are highlighted and underlined. The positions of primers are in accordance with wild-type strain of HAV (GenBank accession number: M14707.1).
4.2.5. Real-time RT-PCR analysis

Real-time PCR was performed with a ViiA™ 7 system (Life Technologies, Burlington, ON, Canada) using primers and probe (Table 4.1): 5’ATAGGGTAACAGCGGCGGATAT3’ (forward), 5’CTCAATGCATCCACTGGAG3’ (reverse) and 5’FAM-CCATTCAACGCGGGAGG-MGB3’ (Taqman probe) (Gardner et al., 2003). The PCR was performed in a 20 µL-reaction consisting of 10 µL of 2 × Taqman Fast Advanced Master Mix (Cat. No. 4444557, Life Technologies, Burlington, ON, Canada), 700 nM of each primer, 250 nM of probe, and 4 µL of cDNA. The reaction mixture was incubated for 2 min at 50°C and for 20 s 95°C, followed by 40 cycles of 1 s at 95°C and 20 s at 60°C. Fluorescence was measured at 60°C for each cycle during the amplification.

4.2.6. Initial processing of each food artificially contaminated with HAV

Around 15 g of green onions (cut into ~3 cm pieces) or 50 g of strawberries were weighed in a clean sterile petri dish and allowed to dry in a biosafety cabinet. One hundred µL of HAV dilution prepared in PBS (pH 7.4, Cat. No. 10010031, Life Technologies, Burlington, ON, Canada) containing \(8.3 \times 10^5\), \(8.3 \times 10^3\), \(8.3 \times 10^1\), or \(8.3 \times 10^0\) PFU viral particles were pipetted evenly onto the surface of green onions or strawberries with 3 µL inoculated at each spot. Each dilution was inoculated onto three independent samples and one uninoculated sample was used as a negative control. After the inoculum was dry, the green onions or strawberries were transferred to a Whirl-pack™ sterile filter bag (Cat. No. 0181269, Fisher Scientific, Ottawa, ON, Canada). Fifty mL of glycine buffer (0.05 M glycine, 0.14 M NaCl, 0.2% (v/v) Tween 20, pH 9.0) were added to the bag. The inoculated viral particles were eluted from the food surface by gently agitating the stomacher bag for 15 min. Then, the eluate
was transferred to a sterile centrifuge tube (50 mL) and centrifuged at 3,500 × g for 15 min to be clarified using a Beckman Coulter Allegra 21 centrifuge with an S4180 rotor (Mississauga, ON, Canada). The supernatant was transferred to a new 50 mL centrifuge tube for further analysis.

New Zealand greenshell mussels (Talley’s Group Ltd.) were purchased from a local grocery store and kept at -80 °C until use. Ten pieces of mussel were aseptically shucked, and the whole tissue of each mussel was removed from the shell and pooled together, which was then finely chopped using sterile scissors (Greening & Hewitt, 2008). The tissue was divided into 5 g portions in a sterile petri dish. One hundred µL of HAV dilutions (8.3 × 10^5, 8.3 × 10^3, 8.3 × 10^1, or 8.3 × 10^0 PFU/inoculum) were inoculated evenly onto the tissue and then incubated for 1 h at room temperature. One portion of mussel tissue without viral inoculum was used as a negative control. The tissue was then transferred into a Whirl-pack™ sterile filter bag. Fifty mL of glycine buffer (0.05 M glycine, 0.14 M NaCl, 0.2% Tween 20, pH 9.0) were added to the bag and then sonicated for 5 min at 40 kHz in a water bath sonicator (FS20, Fisher Scientific, Ottawa, ON, Canada) (Morales-Rayas et al., 2009) and shaken gently for 15 min. The elution fluid was transferred to a sterile 50 mL centrifuge tube and centrifuged at 4,100 × g for 20 min. The supernatant was transferred to a new 50 mL centrifuge tube for further analysis.

Pasteurized partly skimmed milk (2%, Neilson, Canada) was purchased from a local supermarket and stored at 4°C. Forty mL of the milk (in 50 mL centrifuge tube) were inoculated with 100 µL of HAV dilutions prepared in PBS (pH 7.4). The concentration of
HAV in the inoculum was $8.3 \times 10^6$, $8.3 \times 10^4$, $8.3 \times 10^2$, or $8.3 \times 10^1$ PFU/mL, respectively. The artificially contaminated milk was mixed well and then left at room temperature for 1 h. One tube of milk without HAV inoculum was used as a negative control. Six µL of chymosin (CHY-MAX® Extra, Cat. No. 73863, Chr. Hansen, Milwaukee, WI, USA) were added to 40 mL milk and clotting was allowed to take place at 37°C for 20 min. Milk was then centrifuged at 4,100 × g for 20 min at 4°C to precipitate protein. The supernatant was transferred to a new centrifuge tube for further analysis.

4.2.7. Concentration of HAV using PMNPs

Fifty µL of PMNPs were added to the treated food samples to capture HAV through rotating at 10 rpm at room temperature for 20 min. PMNPs were captured by a permanent magnet (Cat. No. 44207-20, Indigo Instrument, Waterloo, Canada) with a surface magnetization of 13,000 gauss for 30 min. Then, the supernatant was discarded and the PMNPs were resuspended in 200 µL of PBS (pH 7.4, Cat. No. 10010031, Life Technologies, Burlington, ON, Canada) and vortexed for 5 min to separate the PMNPs quite well. Then the viral RNA was extracted from the concentrated particles using the QIAamp MinElute Virus Spin Kit (Cat. No. 57704, Qiagen, Toronto, ON, Canada). Two hundred µL of buffer AL and 25 µL of protease in the kit were mixed with the separated PMNPs, and incubated at 56°C for 15 min to lyse the virus capsid. The PMNPs were separated from the lysate by magnetic capture and the lysis buffer containing the released HAV RNA was transferred to a new 1.5 mL Eppendorf centrifuge tube. After the addition of 250 µL of ethanol (100%) and incubation at room temperature for 5 min, the sample was loaded onto the QIAamp MinElute column. The following steps were the same as previously described in Section 4.2.1.
4.3. Results

4.3.1. Real-time PCR of HAV

A two-step real-time RT-PCR was used to amplify serial 10-fold dilutions of HAV cDNA corresponding to a virus titer ranging from $8.3 \times 10^5$ PFU/mL to $8.3 \times 10^0$ PFU/mL. The fluorescence intensity of FAM (6-carboxyfluorescein) was recorded over time during the amplification of each cDNA dilution (Figure 4.3). The amplification profile monitored by fluorescence in real-time PCR is different from that measured by luminescence in LAMP-BART assay (Figures 4.5 – 4.7).

![Figure 4.3. Amplification of HAV cDNA in real-time RT-PCR monitored by the measurement of fluorescence.](image)
4.3.2. RT-LAMP assay of HAV

HAV genomic RNA was extracted from 200 µL of the virus stock containing $8.3 \times 10^6$ PFU/mL of virus strain HM175/24A. The RNA was serially diluted in 10-fold increments, and each dilution was subjected to RT-LAMP assay. The amplified product was analyzed on 1% agarose gel. As is shown in Figure 4.4, amplification product of HAV strain HM175/24A was observed as a ladder-like pattern on a gel. The detection limit of the RT-LAMP was 83 PFU/mL, which was one log higher than that of real-time RT-PCR (8.3 PFU/mL).

![Figure 4.4. Analysis of RT-LAMP amplicon of HAV RNA by 1% agarose gel electrophoresis. Lane M, 2-log DNA ladder (Cat. No. N0469L, New England Biolab); Lane 1, no template control; Lane 2, $10^1$ dilution of RNA; Lane 3, $10^2$ dilution of RNA; Lane 4, $10^3$ dilution of RNA; Lane 5, $10^4$ dilution of RNA; Lane 6, $10^5$ dilution of RNA; Lane 7, $10^6$ dilution of RNA.](image)
4.3.3. Optimization of Mg$^{2+}$ in RT-LAMP-BART assay of HAV

The enzymatic reactions of RT-LAMP-BART were complicated, as shown in Figure 4.1. Optimization of the reaction system is necessary to obtain higher sensitivity. In the current study, the effect of MgSO$_4$ concentrations ranging from 2 mM to 4 mM on RNA-LAMP-BART reaction was determined (Figure 4.5). The effect was tested using two RNA dilutions corresponding to the viral titers of $8.3 \times 10^5$ PFU/mL (Figure 4.5 A) and $8.3 \times 10^3$ PFU/mL (Figure 4.5 B). For both RNA dilutions, the MgSO$_4$ concentration at 2 mM demonstrated the optimal amplification. It was apparent that the time-to-peak of RNA-LAMP-BART reaction performed in the presence of 2 mM MgSO$_4$ was detected earlier than that of the reaction performed with 3 or 4 mM MgSO$_4$. It was also found that with the increase of Mg$^{2+}$ concentration, the reaction sensitivity decreased (Figure 4.5 A & B).

Figure 4.5. Effect of MgSO$_4$ concentrations on RNA-LAMP-BART reaction of HAV. Each curve represents one of three replicates. A, RNA was from $8.3 \times 10^5$ PFU/mL of HAV; B, RNA was from $8.3 \times 10^3$ PFU/mL of HAV.
The effect of MgSO₄ concentrations ranging from 0 mM to 4 mM on cDNA-LAMP-BART reaction was also determined (Figure 4.6). The effect was tested using two cDNA dilutions corresponding to an initial viral titer of $8.3 \times 10^5$ PFU/mL (Figure 4.6 A) and $8.3 \times 10^3$ PFU/mL (Figure 4.6 B). For both cDNA dilutions, the MgSO₄ concentration at 4 mM showed the optimal amplification (Figure 4.6 A & B). The reaction sensitivity increased with the increase of MgSO₄ concentration. A MgSO₄ concentration of 0 mM failed to produce an amplification signal for the cDNA dilution corresponding to a viral titer of $8.3 \times 10^3$ PFU/mL (Figure 4.6 B).

![Figure 4.6. Effect of MgSO₄ concentrations on cDNA-LAMP-BART reaction of HAV. Each curve represents one of three replicates. A, cDNA was from $8.3 \times 10^5$ PFU/mL of HAV; B, cDNA was from $8.3 \times 10^3$ PFU/mL of HAV.](image)

4.3.4. Comparison of HPLC-purified and cartridge-purified primers in RNA-LAMP-BART assay

The effect of primers purified with HPLC and cartridge technology on the efficiency of RNA-LAMP-BART assay was investigated. It was found that HPLC-purified primers produced more sensitive results than cartridge-purified primers (Figure 4.7). Four RNA dilutions were
tested, and for each RNA dilution, the time-to-peak of the reaction using HPLC-purified primers was earlier than that of the counterpart reaction using cartridge-purified primers. For primer synthesis, the longer the sequence, the higher the error rate and a larger number of failed truncated sequences are produced. HPLC can not only remove impurities such as salt and organic solvent, but also eliminate the truncated sequences to a greater extent than the cartridge technique, producing primers with higher yield and purity (http://www.biolegio.com/gb/oligo-synthesis-products/purification/). The inner primers (~ 40 bases) used in LAMP reaction are at least twice as long as the primers (~ 20 bases) used in the PCR method. HPLC technique could guarantee the lower error rate in inner primer sequences.

![Graph](https://via.placeholder.com/150)

**Figure 4.7.** Comparison of HPLC-purified and cartridge-purified primers in RNA-LAMP-BART assay. Four HAV genomic RNA dilutions corresponding to $8.3 \times 10^5$, $8.3 \times 10^4$, $8.3 \times 10^3$, and $8.3 \times 10^2$ PFU/mL of the initial virus titer were tested. The orange line indicated the reactions using HPLC-purified primers. The blue line indicated the reactions using cartridge-purified primers. Each curve represents one of three replicates.
4.3.5. Sensitivity analysis of RNA-LAMP-BART and cDNA-LAMP-BART assay

HAV genomic RNA was extracted from 200 µL of viral stock containing $8.3 \times 10^6$ PFU/mL of HM175/24 A strain. The RNA was serially diluted in 10-fold increments, and each dilution was subjected to the RNA-LAMP-BART assay performed at 55°C for 100 min. A standard curve constructed by plotting the time-to-peak of the luminescence signal against the logarithm of the concentration of 10-fold serial dilutions of virus titer is shown in Figure 4.8. The results showed that the HAV RNA was amplified to at least $10^{-5}$ dilution corresponding to 2.1 PFU/5 µL of the virus. The values indicated that the concentration of HAV with the titer of 83 PFU/mL could be detected by the RNA-LAMP-BART assay within 100 min. This limit of detection was the same as that for conventional RT-LAMP and was one log higher than that obtained using real-time RT-PCR.

![Figure 4.8. Standard curve of RNA-LAMP-BART assay generated by testing the time-to-peak of 10-fold serial dilutions of HAV RNA. Each value is the mean of three independent replicates. Bars show standard deviation. The assay was performed at 55°C for 100 min.](image)
The extracted HAV genomic RNA was reverse transcribed to cDNA, which was then diluted in 10-fold increments. Each dilution was subjected to cDNA-LAMP-BART conducted at 62°C for 100 min. A standard curve constructed by plotting the time-to-peak of the luminescence signal against the logarithm of the concentration of the virus titer is shown in Figure 4.9. The results indicated that the virus with a titer as low as 8.3 PFU/mL, corresponding to 0.083 PFU/4 µL, could be detected by the cDNA-LAMP-BART assay within 100 min. This limit of detection was the same as that obtained using real-time RT-PCR and was one log lower than that of RNA-LAMP-BART assay.

![Figure 4.9. Standard curve of cDNA-LAMP-BART assay made by testing the time-to-peak of 10-fold serial dilutions of HAV cDNA. Each value is the mean of three independent replicates. Bars show standard deviation. The assay was performed at 62°C for 100 min.](image)
4.3.6. Comparison of real-time RT-PCR, RNA-LAMP-BART, and cDNA-LAMP-BART assay for the detection of HAV from different foods

HAV separated and concentrated from green onions, strawberries, mussels, and milk artificially inoculated with different levels of the viral particles was analyzed using real-time RT-PCR, RNA-LAMP-BART, and cDNA-LAMP-BART assay. The detection limit of the three methods varies according to food type (Table 4.2). All food samples uncontaminated with the virus did not give an amplification signal for any of the three detection methods. For green onions, real-time RT-PCR, cDNA-LAMP-BART, and RNA-LAMP-BART assay demonstrated a detection limit of 8.3 PFU/15 g, 8.3 PFU/15 g, and 83 PFU/15 g, respectively. For strawberries, both real-time RT-PCR and cDNA-LAMP-BART were able to detect HAV level as low as 83 PFU/50 g. The RNA-LAMP-BART was apparently inferior to the other two methods in terms of detection limit, with virus at the inoculum level of 83 PFU/50 g or 8.3 PFU/50 g not detectable. HAV at an inoculum level of 8.3 PFU/50 g in strawberries was not detectable by any of the three methods, which might be due to the low efficiency of the virus separation and concentration step or the strong inhibition of the reactions from strawberry compounds. As for mussels and milk, all three molecular methods had a detection limit of 8.3 PFU/sample.
Table 4.2. Comparison of real-time RT-PCR, RNA-LAMP-BART, and cDNA-LAMP-BART assays in detecting HAV in green onions, strawberries, mussels, and milk.

<table>
<thead>
<tr>
<th>Food sample</th>
<th>Method</th>
<th>No. of positive samples / No. of tested samples at inoculation level (PFU) of</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>$10^5$</td>
</tr>
<tr>
<td>Green onion</td>
<td>Real-time RT-PCR</td>
<td>6/6</td>
</tr>
<tr>
<td></td>
<td>RNA-LAMP-BART</td>
<td>6/6</td>
</tr>
<tr>
<td></td>
<td>cDNA-LAMP-BART</td>
<td>6/6</td>
</tr>
<tr>
<td>Strawberry</td>
<td>Real-time RT-PCR</td>
<td>6/6</td>
</tr>
<tr>
<td></td>
<td>RNA-LAMP-BART</td>
<td>6/6</td>
</tr>
<tr>
<td></td>
<td>cDNA-LAMP-BART</td>
<td>6/6</td>
</tr>
<tr>
<td>Mussel</td>
<td>Real-time RT-PCR</td>
<td>6/6</td>
</tr>
<tr>
<td></td>
<td>RNA-LAMP-BART</td>
<td>6/6</td>
</tr>
<tr>
<td></td>
<td>cDNA-LAMP-BART</td>
<td>6/6</td>
</tr>
<tr>
<td>Milk</td>
<td>Real-time RT-PCR</td>
<td>6/6</td>
</tr>
<tr>
<td></td>
<td>RNA-LAMP-BART</td>
<td>6/6</td>
</tr>
<tr>
<td></td>
<td>cDNA-LAMP-BART</td>
<td>6/6</td>
</tr>
</tbody>
</table>

For each food, virus was recovered on three distinct occasions and detection of viral RNA by real-time RT-PCR and RT-LAMP-BART was performed in duplicate resulting in six determinations for each load of virus. Virus inoculum level per food sample was $8.3 \times 10^5$ PFU, $8.3 \times 10^3$ PFU, $8.3 \times 10^1$ PFU, or $8.3 \times 10^0$ PFU.

4.4. Discussion

Molecular biology techniques based on the amplification of viral RNA have been used for the specific and ultrasensitive detection of viruses in foods. The most widely used virus detection method is real-time PCR, a technique developed in the early 1990s (VanGuilder et al., 2008). Since its development, this technique has been improved dramatically. For example, different formats of the instrument have been developed, and the master mix used in the technique has
been optimized to minimize the amplification time and enhance the resistance to inhibitors. In our experiment conducted in this chapter, we used the Taqman Fast Advanced Master Mix (Cat. No. 4444557, Life Technologies, Burlington, ON, Canada) coupled with the ViiA™ 7 system (Life Technologies, Burlington, ON, Canada) to generate results within 40 min. For the study conducted in Chapter 2, we used 1 × Absolute QPCR ROX master mix (Cat. No. AB1138A, Fisher Scientific, Ottawa, Canada) coupled with the ABI 7900HT system (Life Technologies, Burlington, ON, Canada) to generate results within 100 min. As an alternative to PCR technique requiring a thermal cycler, LAMP technique has shown great promise in detecting microbial pathogens in foods. LAMP technique was first reported in 2000 (Notomi et al., 2000). LAMP-BART is a novel technique that was first described in 2010 (Gandelman et al., 2010). Contrary to real-time PCR, this technique is still in the initial-stage of development.

This is the first study of the use of the RT-LAMP-BART method for the detection of foodborne pathogens. HAV was used as a model microorganism to build the method. A one-step RT-LAMP-BART (RNA-LAMP-BART) and a two-step RT-LAMP-BART (cDNA-LAMP-BART) were compared in the current study. The sensitivity of RNA-LAMP-BART and cDNA-LAMP-BART were measured by testing the 10-fold serial dilutions of RNA or cDNA obtained from a viral stock. It was found that 8.3 PFU/mL and 83 PFU/mL of HAV could be detected by cDNA-LAMP-BART and RNA-LAMP-BART, respectively. The cDNA-LAMP-BART showed 1-log higher sensitivity than RNA-LAMP-BART. Different amplification temperature was used in cDNA-LAMP-BART and RNA-LAMP-BART, with 62°C and 55°C for the former and latter, respectively. The Lumora Ltd., who developed the
LAMP-BART technique, recommended 55°C as the reaction temperature for RNA-LAMP-BART because the reverse transcriptase used in their reaction as not stable above 55°C, thus we did not try other temperatures to investigate the effect of higher temperature on the sensitivity of the assay. This lower amplification temperature might contribute to the lower sensitivity of RNA-LAMP-BART in our experiment. Yoneyama et al (2007) used a RT-LAMP method to detect HAV by measuring the turbidity of the reaction mixture in real-time due to the formation of magnesium pyrophosphate. In their study, 0.6 focus forming units (FFU)/reaction of HAV, corresponding to $1.5 \times 10^1$ FFU/mL of the virus, was detectable. In our study, 2.1 PFU/reaction of HAV, corresponding to $8.3 \times 10^1$ PFU/mL of the virus, was detectable by RNA-LAMP-BART. Therefore, the sensitivity of RNA-LAMP-BART assay developed in our study is comparable to the reported study. The sensitivity of cDNA-LAMP-BART and real-time RT-PCR used in our study were the same to be 0.083 PFU/reaction, corresponding to the virus titer of 8.3 PFU/mL. The value was lower than 0.5 PFU/reaction reported by Jothikumar et al. (2005) for using Taqman real-time PCR to detect HAV RNA, lower than 1 PFU/reaction reported by Abd El Galil et al. (2004) for using molecular-beacon real-time RT-PCR to detect HAV RNA, and lower than 2 PFU/reaction reported by Jean et al. (2002) for using nuclei acid sequence-based amplification (NASBA) method to detect HAV.

The optimization of reaction system is necessary for sensitive detection. We optimized Mg$^{2+}$ concentration in the current study to improve the sensitivity of RT-LAMP-BART assay. The concentration of Mg$^{2+}$ has also been optimized in previous studies. Liu et al. (2013) found that 5.75 mM of Mg$^{2+}$ was optimal in detection of *Leifsonia xyli* subsp. *xyli* in sugarcane using LAMP. Liu et al. (2011) reported that the optimal concentration of Mg$^{2+}$ in using LAMP to
detect *Bacillus cereus* in milk was 2 mM. Nie (2005) reported that 6 mM to 8 mM of Mg$^{2+}$ were optimal in detecting *Potato virus Y* using LAMP. Aside from Mg$^{2+}$ concentration, other factors influencing the sensitivity of LAMP reaction, such as primer concentration, dNTP (deoxyribonucleotide triphosphate) concentration, enzyme concentration, reaction temperature, and reaction time, were also optimized in these studies. However, Mg$^{2+}$ concentration was found to be the most critical factor when optimizing the LAMP reaction (Liu et al., 2013; Liu et al., 2011). Magnesium ions are important to the RT-LAMP-BART reaction. It was reported that magnesium ions were the cofactors of the enzymes used in the RT-LAMP-BART assay, such as reverse transcriptase, *Bst* polymerase, ATP sulphurylase, and luciferase, and had a role in the activation of enzymes by changing their conformation (Cowan, 2002). ATP can only become active upon binding with Mg$^{2+}$ (Cowan, 2002; Nakatsu et al., 2006), increasing the production of luminescence. Thus, inadequate Mg$^{2+}$ in the reaction could lead to inactive enzymes and ATP and hence slow down chemical reaction rates. Magnesium ions could also chelate dNTPs (Goto et al., 2009) and mediated the replication of DNA (Cowan, 2002). Therefore, excessive Mg$^{2+}$ in the reaction could also inhibit the process of chemical reactions in LAMP-BART. In our study, 2 mM Mg$^{2+}$ and 4 mM Mg$^{2+}$ was the optimal concentration for RNA-LAMP-BART and cDNA-LAMP-BART, respectively.

In the current study, the primers used in LAMP and real-time RT-PCR for the detection of HAV were selected from the same conserved region (5’-UTR) of HAV genome. The specificity of the LAMP primers used for HAV detection has been examined by testing several genotypes of HAV and other enteric viruses, and they were found to only amplify HAV target sequences (Yoneyama et al., 2007). For LAMP primers, the inner primers generate hairpin
loops, and the outer primers displace the DNA strands. Aside from inner and outer primers, the use of loop primers (Nagamine et al., 2002) or stem primers (Gandelman et al., 2011) could accelerate the speed and sensitivity of the reaction. We found that the sensitivity of the assay could also be improved by primer quality. HPLC-purified primers were used in some reports (Luo et al., 2011; Yang et al., 2011), and there were others using cartridge-purified primers (Yoda et al., 2007) in the LAMP assay. By comparing the results from HPLC-purified and cartridge-purified primers, we found that HPLC-purified primers demonstrated higher sensitivity of LAMP-BART reaction.

HAV seeded on green onions, strawberries, mussels, or in milk, was detected using RNA-LAMP-BART and cDNA-LAMP-BART. The sensitivity of cDNA-LAMP-BART and real-time RT-PCR was comparable in testing HAV in each food (Table 4.2). RNA-LAMP-BART assay showed better performance in testing HAV in mussels and milk than in green onions and strawberries, which might be due to the fact that primers purified with different methods were used when detecting HAV in different foods. The cartridge-purified primers were used for testing HAV in green onions and strawberries, while HPLC-purified primers were used for testing the virus in mussels and milk. It was found that the RNA-LAMP-BART method demonstrated 1-log higher sensitivity in testing HAV RNA from food samples (mussels and milk) (Table 4.2) than that from viral stock (Figure 4.8). The observation was partly because of different amplification time used in our experiment. The RNA-LAMP-BART was performed for 100 min in testing RNA purified from a viral stock, while 130 min were used for testing HAV concentrated from each food sample. The light signal of RT-LAMP-BART for virus at an inoculum level of 8.3 PFU in mussels and milk appeared in more than 100 min.
The amplification time of the RT-LAMP-BART assay (130 min) was longer than that of real-time RT-PCR (40 min) used in the current study. But, the limit of detection of LAMP-BART assay developed in the current study is able to satisfy the need for diagnostic applications in a food microbiological laboratory, because HAV infectious dose is between 10 and 100 viral particles (Yezli & Otter, 2011). In future, the components of the master mix used for the RT-LAMP-BART reaction should be optimized to further improve the sensitivity, speed, and robustness of the method.

Although the amplification time of RT-LAMP-BART was longer than that of real-time RT-PCR in the current study, RT-LAMP-BART has some advantages over real-time PCR. The instrument used for RT-LAMP-BART and the software used for data interpretation is simple, because RT-LAMP-BART relies on the time-to-peak of light output not the absolute light intensity (Gandelman et al., 2010). An instrument containing a heating block capable of controlling temperature and a photodiode or a charge-coupled device (CCD) camera capable of detecting light can satisfy the requirements of the method (Gandelman et al., 2010). Real-time PCR is based on the detection of absolute fluorescence intensity during a thermal cycling reaction and requires an instrument consisting of a laser or light-emitting diode (LED) for emitting a broad spectrum of light, filters for selecting the excitation and emission wavelength of specific fluorophores, mirrors for reflecting light, a photodiode, CCD or photomultiplier tube for detecting emitted light, and a device for heating and cooling the reaction plate. The instrument can only be operated by trained personnel and needs to be calibrated frequently to guarantee accurate experimental results. However, any person can operate the Bison system (Lumora Ltd.) used for LAMP-BART after a short training session and the maintenance of the
instrument is simple and easy. The instrument software used for real-time PCR protocol setup, data collection and data analysis is much more complicated than that for LAMP-BART. Due to the isothermal character of the reaction, the LAMP instrument has the potential to be miniaturized for convenient carrying and hence used in field applications. Various formats of portable devices have been designed to perform the LAMP reaction in remote areas where resources are limited. A portable ESE Quant tube scanner has been used for detection of fluorescence signal of SYBR Green I in the amplification of the genome of *Vibrio parahaemolyticus* and malaria using LAMP (Lucchi et al., 2010; Surabattula et al., 2013; Yi et al., 2014). It was also reported that the LAMP reaction could be performed on a silicon chip for the detection of virulence genes of *L. monocytogenes*, *E. coli*, and *Salmonella* (Duarte et al., 2013). Microfluidic devices have also been developed for the detection of foodborne pathogens such as *C. jejuni*, *Shigella*, *S. Typhimurium*, and *Vibrio cholerae* using the LAMP technique (Hsieh et al., 2012; Tourlousse et al., 2012). All these portable devices are based on fluorescence detection. Because luminescence detection is much simpler than fluorescence, the portable device for LAMP-BART will be easier to design. Lumora Ltd. has manufactured a portable instrument capable of testing eight samples at one time to enable the LAMP-BART technique to be performed in the field (Kiddle et al., 2012). The less expensive, energy-saving, and easy-to-use portable device might be produced for specific foodborne virus detection using LAMP-BART in the near future. With the great potential for the instrument to be miniaturized, this technique is promising in detection of foodborne pathogens in future.
4.5. Conclusions

RT-LAMP-BART was employed for the detection of HAV concentrated from different foods including green onions, strawberries, mussels, and milk. The sensitivity of the assay was determined by factors such as Mg$^{2+}$ concentration and primer quality. The Mg$^{2+}$ concentration was optimized for RNA-LAMP-BART and cDNA-LAMP-BART reaction. The optimal Mg$^{2+}$ concentration was 2 mM for RNA-LAMP-BART and 4 mM for cDNA-LAMP-BART. Compared with cartridge-purified primers, HPLC-purified primers could greatly improve the sensitivity of the LAMP-BART method. The results of cDNA-LAMP-BART were comparable with two-step real-time RT-PCR for detecting HAV in different foods. The detection limit of the two methods for HAV in green onions, strawberries, mussels, and milk was 8.3 PFU/15 g, 83 PFU/50 g, 8.3 PFU/5 g, and 8.3 PFU/40 mL, respectively. RNA-LAMP-BART showed less sensitivity than cDNA-LAMP-BART in detecting HAV in green onions and strawberries.
Chapter 5: APPLICATION OF Q-BETA REPLICASE REACTION ASSAY FOR THE DETECTION OF HEPATITIS A VIRUS

5.1. Introduction

The Qβ replicase reaction assay is based on the nucleic acid hybridization and Qβ replicase amplification (Lizardi et al., 1988). In contrast to PCR aiming to amplify the target sequence, the Qβ replicase reaction assay focuses on amplifying probe molecules that are specifically hybridized to the target (Blok & Kramer, 1997; Stefano et al., 1997). In the Qβ replicase assay, a small, naturally occurring MDV-1 (midivariant) template RNA, in which target-complementary sequence elements have been inserted, is used as a hybridization probe (reporter probe) (Lizardi et al., 1988). After isolation of the probe:target hybrid, the reporter probe is amplified by Qβ replicase. Since the daughter strand of the replicated product is also a good template for Qβ replicase, both RNA strands accumulate exponentially (Kramer & Lizardi, 1989). The Qβ replicase reaction assay is an isothermal amplification technique with the reaction conducted at a constant temperature, 37°C. Thus, a thermal cycler used to control temperature in PCR is not needed in this method. No primer is needed in the Qβ replicase reaction (Tyagi et al., 1996). The amplification of RNA can be monitored by testing the fluorescence of a RNA-intercalating dye such as SYBR Green II that does not affect the kinetics of replication (Bansho et al., 2012). The time at which fluorescence is detectable above the background is called response time (Burg et al., 1995). There is an inverse and linear proportional relationship between the target concentration and the response time.
The Qβ replicase reaction assay has been reported not to be sensitive to inhibitors in sputum specimens for *Mycobacterium tuberculosis* detection, while the performance of PCR was significantly compromised (An et al., 1995). In addition to *Mycobacterium tuberculosis*, the assay has been applied for the detection of *Chlamydia trachomatis* (Shah et al., 1994), *Mycobacterium avium* complex, *Mycoplasma pneumoniae*, *Pneumocystis carinii*, *Legionella pneumophila* (Stone et al., 1996), and human immune deficiency virus (HIV) (Axelrod et al., 1991; Lizardi et al., 1988; Lomeli et al., 1989; Tyagi et al., 1996) in clinical samples.

Due to the rapidity, sensitivity, and strong tolerance to inhibitors of the Qβ replicase reaction assay, the method was chosen in the current study for detecting foodborne viruses. In the current study, we have synthesized MDV-based recombinant RNA molecules as reporter probes for HAV detection. The inserted RNA sequence was selected from the conserved region (5’ UTR) of HAV genome. The recombinant RNAs could be amplified exponentially by Qβ replicase. In the Qβ replicase assay, residual non-hybridized reporter probes could be amplified in parallel with the hybridized probes, causing a high background signal. A new strategy was proposed in this report to solve the problem. However, the amplification signal from the no template control is a tricky problem that limits the application of the method.

5.2. Materials and methods

5.2.1. Materials

Qβ replicase (Cat. No. QB501250) was purchased from Epicentre Biotechnologies (Madison, Wisconsin, USA). Plasmid pUC-MDV-LR (Figure 5.1) carrying the segment corresponding to MDV-poly (+) RNA downstream of the T7 promoter sequence was kindly provided by Dr.
Norikazu Ichihashi at Osaka University in Japan. The plasmid also contained a SmaI restriction site at the 3’ end of MDV-poly sequence, and a BglII site within the MDV-poly RNA sequence for cloning (Lizardi et al., 1988). Two oligonucleotides, 5’agatctTGCAAGGTGACGTTCACATCTG1GCATCCACATTATAGACTCCTACAGCTCCATagatct 3’, and 5’agatctCCTCGAGTAGAGCGCGCGTGCAAGGTGACGTTCACATCTG1GCCTCCACATTATAGACTCCTACAGCTCCATagatct3’, were synthesized by DNA2.0 Inc. (Menlo Park, CA, USA). Both nucleotides contained a probe (underlined sequence) that was complementary to the nucleotide 279 – 333 of HAV HM175/24A (GenBank accession number M59810.1). For the shorter sequence (67 nt), the HAV probe (55 nt) was flanked by a BglII site (lower case letters). And for the longer one (96 nt), in addition to BglII sites, the probe was also flanked by some spacer elements (29 nt).

Figure 5.1. The structure of plasmid pUC-MDV-LR (Inokuchi et al., 1994; Lizardi et al., 1988; Nakaishi et al., 2002). The heavy black line represents MDV-1 cDNA.
5.2.2. Construction of plasmids for synthesizing HAV-reporter probes

5.2.2.1. Ligation of inserts with plasmid pUC-MDV-LR

The two synthesized oligonucleotides were inserted into BglII site of the plasmid pUC-MDV-LR, which was located between nucleotides 63 and 64 of the plus-strand of MDV-1 (Miele et al., 1983). In order to achieve this, both the double-stranded oligonucleotides and the plasmid pUC-MDV-LR were digested with BglII (Cat. No. R0144S, New England Biolabs, Whitby, ON, Canada). The reaction conditions for BglII digestion of the plasmid and insertion sequences are shown in Table 5.1. Molecular-grade water (Cat. No. 10977-015, Life Technologies, Burlington, ON, Canada) was used to adjust the final volume of the reaction to be 60 µL. After digestion, the pUC-MDV-LR was purified with the QIAquick PCR Purification Kit (Cat. No. 28104, Qiagen, Toronto, ON, Canada) according to the manufacturer’s instructions. Briefly, 300 µL of buffer PB were mixed with the digested plasmids and then the mixture was loaded onto the QIAquick column. After centrifugation at 17,900 × g for 60 s using a Beckman Coulter microfuge 22R (Mississauga, ON, Canada), the flow-through was discarded and the column was washed with 750 µL of buffer PE by centrifugation again for 60 s. After centrifugation of the column for additional 60 s, 50 µL of buffer EB (10 mM Tris-HCl, pH 8.5) were used to elute DNA. Then, the purified plasmid pUC-MDV-LR was dephosphorylated with Calf Intestinal Alkaline Phosphatase (Cat. No. M0290S, New England Biolabs, Whitby, ON, Canada) at 37°C for 2 h, and purified with the QIAquick PCR Purification Kit again. The purified plasmids were stored at -20°C for further analysis.
Table 5.1. BglII digestion system for pUC-MDV-LR and insertion sequences.

<table>
<thead>
<tr>
<th>Reaction conditions</th>
<th>pUC-MDV-LR</th>
<th>Insertion sequences</th>
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<tbody>
<tr>
<td>DNA usage (µg)</td>
<td>6</td>
<td>1</td>
</tr>
<tr>
<td>BglII volume (µL)</td>
<td>5</td>
<td>8</td>
</tr>
<tr>
<td>10 × NEBuffer 3 (µL)</td>
<td>6</td>
<td>10</td>
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<tr>
<td>Total reaction volume (µL)</td>
<td>60</td>
<td>100</td>
</tr>
<tr>
<td>Reaction time (h)</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>Reaction temperature (°C)</td>
<td>37</td>
<td>37</td>
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The digested insertion sequences were purified with the QIAquick Nucleotide Removal Kit (Cat. No. 28304, Qiagen, Toronto, ON, Canada) in accordance with the manufacturer’s instructions. Briefly, 1 mL of buffer PN was mixed with 100 µL of BglII digested sequences. The sample was loaded onto the QIAquick column and centrifuged for 1 min at 8,000 × g using a Beckman Coulter microfuge 22R (Mississauga, ON, Canada). The flow-through was discarded and the column was washed with 750 µL of buffer PE through centrifugation at 8,000 × g for 1 min. After centrifugation of the column for additional 1 min at 17,900 × g, the bound DNA was eluted with 50 µL of buffer EB (10 mM Tris-HCl, pH 8.5) and stored at -20°C.

The purified insertion sequences were ligated with the linearized pUC-MDV-LR using the Quick Ligation Kit (Cat. No. M2200S, New England Biolabs, Whitby, ON, Canada). The 20-µL reaction consisted of 10 µL of 2 × Quick Ligation Buffer, 1 µL of Quick T4 DNA Ligase, 50 ng of plasmid, and 4-fold molar excess of inserts. The reaction mixture was incubated at 25°C for 5 min and then chilled on ice. The ligated product was stored at -20°C.
5.2.2.2. PCR analysis

For inserting an oligonucleotide to a plasmid using a single restriction enzyme, it should be verified that the insert was cloned in the correct orientation. The two recombinant plasmids were tested by PCR. The primers were designed by Oligo7.0 software. Forward primer was: 5’GTGTCCTTTAATAGTACCT3’, which was selected from the inserted sequence, and reverse primer was: 5’TATGACCATTACGCAAG3’, which was selected from the plasmid pUC-MDV-LR. The lengths of the PCR amplicons for the recombinant plasmids with longer and shorter insertion sequences were 273 bp and 264 bp, respectively. The PCR reaction mixture consisted of 45 µL of PCR supermix (Cat. No. 10572-014, Life Technologies, Burlington, ON, Canada), 200 nM of each primer, and 1 µL of plasmid. The reaction mixture was incubated at 95°C for 5 min, followed by 34 cycles of 30 s at 95°C, 30 s at 53.5°C and 20 s at 72°C, followed by an extension at 72 °C for 10 min, and finally kept at 4 °C. Ten µL of PCR product were visualized by 1% agarose gel electrophoresis as previously described in Section 4.2.2.

5.2.2.3. Plasmid transformation

Plasmids were transformed into One Shot Top10 chemically competent *E. coli* cells (Cat. No. C4040-10, Life Technologies, Burlington, ON, Canada) following the manufacturer’s instructions. Briefly, three µL of the recombinant plasmid were mixed with the competent cells and incubated on ice for 30 min, heat-shocked for 30 s at 42°C, and then chilled on ice for 2 min. Two hundred and fifty µL of S.O.C. medium were added to the tube, followed by shaking incubation at 37°C and 225 rpm for 1 h. One hundred µL of the transformed cells were spread on LB agar plate (Cat. No. BP9724500, Fisher Scientific, Ottawa, Canada)
containing 100 µg/mL ampicillin. The plates were incubated at 37°C for 16 – 20 h. Then, colony PCR was conducted to screen for successful transformants. Briefly, about 25 single colonies on the plates were randomly picked and suspended in 80 µL of molecular-grade water (Cat. No. 10977-015, Life Technologies, Burlington, ON, Canada) followed by heating at 98°C for 5 min to lyse *E. coli* cells. After centrifugation at 16,000 × g for 2 min using a Beckman Coulter microfuge 22R (Mississauga, ON, Canada), 3 µL of the supernatant were tested by PCR as described previously in Section 5.2.2.2.

Plasmids were propagated and isolated from the positive colonies using the QIAprep Spin Miniprep Kit (Cat. No. 27104, Qiagen, Toronto, ON, Canada). A single colony on a fresh plate was inoculated into 5 mL of LB broth (Cat. No. BP9723500, Fisher Scientific, Ottawa, Canada) containing 100 µg/mL ampicillin, followed by shaking incubation at 37°C and 225 rpm for 16 h. The bacterial cells were harvested by centrifugation at 5,400 × g for 10 min at 4°C using a Beckman Coulter J2-MC centrifuge (Mississauga, ON, Canada). The pelleted cells were resuspended in 250 µL of buffer P1 and transferred to a microcentrifuge tube. Two hundred and fifty µL of buffer P2 were added to the tube and the sample was mixed thoroughly by inverting the tube 4 – 6 times. Then, 350 µL of buffer N3 were added and the sample was mixed thoroughly again by inverting the tube 4 – 6 times, followed by centrifugation at 17,900 × g for 10 min using a Beckman Coulter microfuge 22R (Mississauga, ON, Canada). The supernatant was loaded onto the QIAprep spin column and the column was centrifuged at 17,900 × g for 1 min. Then, the column was sequentially washed with 0.5 mL of buffer PB and 0.75 mL of buffer PE. After centrifugation for additional 1 min to remove residual wash buffer, the DNA bound on the column was eluted using 50 µL of buffer EB and
stored at -20°C. The presence of the inserts in the plasmids was confirmed by sequence analysis conducted at Laboratory Services, University of Guelph (Guelph, ON, Canada).

5.2.3. RNA transcription

The plasmid pUC-MDV-LR, the recombinant plasmids, pUC-MDV-HLP (HAV long probe) with longer insertion, and pUC-MDV-HSP (HAV short probe) with shorter insertion, were digested with SmaI (Cat. No. R0141S, New England Biolabs, Whitby, ON, Canada) at 25°C overnight. The digested plasmids were purified with the QIAquick PCR Purification Kit (Cat. No. 28104, Qiagen, Toronto, ON, Canada) as previously described in Section 5.2.2.1. Then, 1 mg of the linearized plasmid was mixed with 1 µL of T7 RNA polymerase (Cat. No. AM2082, Life Technologies, Burlington, ON, Canada), 2 µL of 10 × transcription buffer, 500 nM of each ATP, UTP, GTP and CTP, and 1 µL of RNase inhibitor (Cat. No. AM2694, Life Technologies, Burlington, ON, Canada) in a 20-µL reaction. The assembled reagents were incubated at 37°C for 1 h for RNA transcription, and then were incubated at 37°C for 15 min after the addition of 1 µL of DNase I (Cat. No. AM2238, Life Technologies, Burlington, ON, Canada) to cleave DNA. The transcribed RNA was purified with the RNeasy Mini Kit (Cat. No. 74104, Qiagen, Toronto, ON, Canada) according to the manufacturer’s instructions. Briefly, the sample volume was adjusted to 100 µL with RNase-free water. Three hundred and fifty µL of buffer RLT and 250 µL of ethanol (100%) were sequentially added to the tube and mixed well. The sample (700 µL) were loaded onto the RNeasy Mini spin column and the column was centrifuged at 8,000 × g for 15 s using a Beckman Coulter microfuge 22R (Mississauga, ON, Canada). Five hundred µL of buffer RPE were used to wash the column twice by centrifugation. Finally, 50 µL of RNase-free water were used to elute RNA and the
purified RNA was stored at -80°C. The concentration of RNAs was tested by a NanoDrop 1000 spectrophotometer (Thermo Scientific), and the size was tested by 2% agarose gel electrophoresis. For performing the agarose gel electrophoresis, 1 µL of RNA was mixed with 5 µL of 2 × RNA loading dye (Cat. No. B0363S, New England Biolabs, Whitby, ON, Canada), and 4 µL of RNase-free water. The mixture (10 µL) was heated at 65°C for 5 min, chilled on ice, and loaded on 2% agarose gel for electrophoresis as described in Section 4.2.2.

5.2.4. RNA replication by Qβ replicase

RNA templates were diluted in RNase-free water (Cat. No. 10977-015, Life Technologies, Burlington, ON, Canada). Three µL of the appropriate dilution of RNA were incubated with 1.5 µg of Qβ replicase at 37°C in a reaction system (30 µL) containing 1 × TA buffer (pH 7.8, Cat. No. TA6160, Epicentre Biotechnologies, Madison, WI, USA), 1.2 mM each of GTP, ATP, CTP, and UTP (Cat. No. R344NT, Epicentre Biotechnologies, Madison, WI, USA), and 1 × concentration of SYBR Green II (Cat. No. S7564, Life technologies, Burlington, ON, Canada) prepared with Triton X-100 (Cat. No. HFH-10, Life Technologies, Burlington, ON, Canada) in a 96-well plate (Cat. No. 3993, Corning, Tewksbury, MA, USA) for 60 min. The increment of fluorescent signal was monitored by a VICTOR³ instrument (Perkin Elmer Inc., Woodbridge, ON, Canada) at 30 s intervals using excitation and emission wavelengths of 490 nm and 530 nm, respectively. The reaction without a template was used as a negative control and the reaction incorporating MDV-poly (MDV-1 with restriction endonuclease sites) was used as a positive control. Two µL of each amplified RNA were also analyzed by 2% agarose gel electrophoresis as described previously in Section 5.2.3.
5.2.5. Qβ replicase reaction assay protocol

The schematic chart of the experimental design using the Qβ replicase reaction for the detection of HAV is shown in Figure 5.2. The assay protocol was modified from Tyagi et al. (1996). Purified HAV genomic RNA, $10^{11}$ molecules of the first capture probe, 5’-biotin-(NH$_2$)$_{10}$-AACAACCTCAACCATATCCCGCTGTTACCCCTATCCAG-3’ that was complementary to nucleotide 427 – 465 of HAV HM175/24A, and $10^{10}$ molecules of HAV-reporter probes were mixed together in 100 µL of buffer A containing 2 M guanidine thiocyanate (Cat. No. 50980, Sigma-Aldrich, Oakville, ON, Canada), 400 mM Tris-HCl (Cat. No. 93363, Sigma-Aldrich, Oakville, ON, Canada), pH 7.5, 5 mg/mL of sodium N-lauroylsarcosine (Cat. No. 61743, Sigma-Aldrich, Oakville, ON, Canada), 5 mg/mL BSA (Cat. No. B6917, Sigma-Aldrich, Oakville, ON, Canada), and 80 mM EDTA (Cat. No. 03677, Sigma-Aldrich, Oakville, ON, Canada), in wells of a 96-well plate. Following the 60-min hybridization at 37°C, 20 µL of streptavidin-derivatized paramagnetic particles (Cat. No. Z5481, Promega Biosciences, Madison, WI, USA) were added to the reaction. After mixing, the plate was incubated at 37°C for 10 min to capture the ternary hybrids (capture probe-target-reporter probe). The particles containing the captured hybrids were collected onto the sides of wells by using a magnetic separator (Cat. No. S1511S, New England Biolabs, Whitby, ON, Canada) for 2 min, and the supernatants were removed by aspiration. Most of the non-hybridized reporter probes were removed by washing the paramagnetic particles three times with buffer A. Then, $10^{11}$ molecules of the second capture probe, 5’-biotin-(NH$_2$)$_{10}$-AGGAGTCTAAATTGGGACACAGATGTTTG-3’, which was the same as the nucleotide 289 – 319 of HAV HM175/24A, were added to the reaction to hybridize to the residual non-hybridized reporter probes at 37°C for 30 min. The paramagnetic particles were washed four
times with buffer B containing 5 mM MgCl$_2$ (Cat. No. BP214-500, Fisher Scientific, Ottawa, ON, Canada), 66 mM Tris-HCl, pH 7.5, 1 mM ATP, 0.5 mg/mL IGEPAL CA-630 (Cat. No. 18896, Sigma-Aldrich, Oakville, ON, Canada), and 1 mM dithiothreitol (Cat. No. 43815, Sigma-Aldrich, Oakville, ON, Canada) to remove guanidine thiocyanate. After the last wash, the particles were suspended in 50 µL of buffer B containing 1 unit of RNase H (Cat. No. M0297S, New England Biolabs, Whitby, ON, Canada) and incubated at 37°C for 10 min to lyse the RNA hybridizing to DNA (Crooke et al., 1995; Inoue et al., 1987; Nakamura et al., 1991; Stein & Hausen, 1969), which broke the non-hybridized reporter probes into two parts that could not be amplified by Qβ replicase (Tyagi et al., 1996). At the same time, the reporter probe-target hybrids were released from the ternary hybrids. The paramagnetic particles were collected on the sides of the wells by a magnetic separator, and the supernatant containing the released reporter probe-target hybrids were transferred to a clean set of wells. Then, 3 µL of the released hybrid complex were amplified by Qβ replicase and the fluorescence signal was monitored as described previously by a VICTOR$^3$ instrument.
Figure 5.2. Schematic chart showing the process of using the Qβ replicase reaction assay to detect virus genome. (A) & (B) hybridization of reporter probes and capture probes with target RNA; (C) capture of ternary hybrids by streptavidin-coated paramagnetic particles in a magnetic field; (D) most of non-hybridized reporter probes are washed away; (E) the second capture probes are added to hybridize to residual non-hybridized reporter probes; (F) RNase H is added to digest RNA that hybridizes to DNA, breaking residual reporter probes and releasing reporter probe-target duplex from paramagnetic particles; (G) capture probes are removed; (H) reporter probes are amplified exponentially by Qβ replicase.
5.3. Results

5.3.1. Construction of recombinant plasmids

The cDNA sequences of two different HAV reporter probes, MDV-HLP and MDV-HSP, were inserted into the BglII site of the plasmid pUC-MDV-LR. After ligation of the linearized plasmid and insertions, the direction of the insertions was tested by PCR. As is shown in Figure 5.3, for the plasmid pUC-MDV-LR without any insertion, there was no amplified product (Lane 1 & 2). The amplicon length of the plasmid pUC-MDV-HLP with 90 bp insert was 273 bp (Lane 3 – 6), and it was 264 bp for the plasmid pUC-MDV-HSP with 61 bp insert (Lane 7 – 10). This indicated that the ligated products contained the expected recombinant plasmids. Each ligated product was transformed into One Shot Top10 *E. coli* competent cells. For each plasmid, around 25 colonies on LB agar plate were tested by PCR. As is shown in Figure 5.4, 5 (colony 14, 16, 21, 22, and 25) out of 22 colonies harbored the expected recombinant plasmid pUC-MDV-HLP, and 12 (colony 6, 8, 9, 10, 13, 14, 18, 19, 21, 22, 26, 28) out of 26 colonies contained the expected recombinant plasmid pUC-MDV-HSP (Figure 5.5). The plasmid pUC-MDV-HLP was propagated and isolated from the colony 14, and the plasmid pUC-MDV-HSP was propagated and isolated from the colony 21. The sequences of the plasmids were analyzed.
Figure 5.3. Agarose gel electrophoretogram of PCR for recombinant plasmids. M: quick-load 2-log DNA ladder (Cat. No. N0469L, New England Biolabs); Lanes 1 & 2: PCR of pUC-MDV-LR. Lanes 3-6: PCR of ligated product with 90 bp insertion; Lanes 7-10: PCR of ligated product with 61 bp insertion.

Figure 5.4. Agarose gel electrophoretogram of colony PCR for the plasmid pUC-MDV-HLP. M: quick-load 2-log DNA ladder (Cat. No. N0469L, New England Biolabs); Lanes 1-3: PCR of colonies in the negative control plate, where the colonies contained the ligated vector without any insertion; Lanes 4 - 25: PCR of colonies from plates, where the colonies contained the plasmid with 90 bp insertion.
Figure 5.5. Agarose gel electrophoretogram of colony PCR for the plasmid pUC-MDV-HSP. M: quick-load 2-log DNA ladder (Cat. No. N0469L, New England Biolabs); Lanes 1 & 2: PCR of colonies in the negative control plate, where the colonies contained the ligated vector without any insertion; Lanes 3 - 28: PCR of colonies from plates, where the colonies contained the plasmid with 61 bp insertion.

5.3.2. RNA transcription

Three plasmids, pUC-MDV-LR, pUC-MDV-HLP, and pUC-MDV-HSP, were digested by SmaI. The size of pUC-MDV-LR (2,934 bp), pUC-MDV-HLP (3,024 bp), and pUC-MDV-HSP (2,995 bp) was tested by agarose gel electrophoresis (Figure 5.6). And then the linearized plasmids were transcribed with T7 RNA polymerase. Three RNAs, MDV-poly, MDV-HLP, and MDV-HSP, were obtained. MDV-HLP and MDV-HSP were complementary to exactly the same region of HAV genome except that MDV-HLP was designed with spacer elements flanking the HAV-complementary sequence. The function of the spacers was reported to be capable of improving the hybridization and replication efficiency of the MDV-reporter probe (Burg et al., 1996). The expected sizes of MDV-poly, MDV-HSP, and MDV-HLP, were 244, 305, and 334 nt, respectively. The size of each RNA was confirmed by agarose gel electrophoresis (Figure 5.7). The concentration of RNAs tested by the Nanodrop 1000
Spectrophotometer was 69 ng/µL for MDV-poly, 117.7 ng/µL for MDV-HSP, and 124.8 ng/µL for MDV-HLP. According to the equation,

\[
\frac{(\text{ug} \times 10^6) (1 \times 10^{12} \text{ pmol/mol})}{(340 \text{ g/mol})(\text{ # of bases })} = \text{pmol of nucleic acid} \quad \text{(Olmos et al., 2005), in which}
\]

1 pmol = 6.02 × 10^{11} molecules, the concentration of MDV-poly, MDV-HSP, and MDV-HLP were 5.01 × 10^{11}, 6.83 × 10^{11}, and 6.62 × 10^{11} copies/µL, respectively. The secondary structures of MDV-poly, MDV-HSP, and MDV-HLP predicted by the software DNAman 6.0 are shown in Figures 5.8, 5.9, and 5.10, respectively. The unique secondary structure of MDV-1 (Figure 1.5) was maintained in the secondary structure of MDV-HLP and MDV-HSP, ensuring that the two reporter probes could be amplified by Qβ replicase (Lizardi et al., 1988).

Figure 5.6. Agarose gel electrophoretogram of linearized plasmids. M: quick-load 2-log DNA ladder (Cat. No. N0469L, New England Biolabs); Lanes 1: plasmid pUC-MDV-LR; Lanes 2: plasmid pUC-MDV-HSP; Lanes 3: plasmid pUC-MDV-HLP.
Figure 5.7. Agarose gel electrophoresis of transcribed RNAs. M: low range ssRNA ladder (Cat. No. N0364S, New England Biolabs); Lane 1: positive control (334 nt) of the T7 transcription kit (Cat. No. AM1312, Life Technologies, Burlington, ON, Canada); Lane 2: MDV-poly (244 nt); Lane 3: MDV-HLP (334 nt); Lane 4: MDV-HSP (305 nt).

Figure 5.8. The secondary structure of MDV-poly. The multiple cloning sites are shown in the circled area.
Figure 5.9. The secondary structure of MDV-HSP. The nucleotides inside the circled areas are BglII sites. The short sequence between the two BglII sites is HAV specific insertion.

Figure 5.10. The secondary structure of MDV-HLP. The nucleotides inside the circled areas are BglII sites. The short sequence between the two BglII sites is HAV specific insertion.
5.3.3. RNA replication by Qβ replicase

A profile of fluorescence signal recorded by the VICTOR³ instrument with the amplification of MDV-poly, MDV-HLP, and MDV-HSP by Qβ replicase is shown in Figure 5.11. Both reporter probes could be replicated in our experiment. At the same initial template concentrations (10⁶ molecule/reaction), the response time of MDV-HLP (23.9 min) was smaller than MDV-HSP (25.3 min) (Figure 5.11). Although the difference was not significant ($P = 0.224$), the results were in good agreement with previous reports that the addition of spacers to a reporter probe could help to improve the amplification efficiency of the reporter probe (Burg et al., 1996). The response times of two reporter probes were significantly higher ($P = 0.03$) than MDV-poly (18.4 min), which might be because the tertiary RNA confirmation of MDV-poly was changed after probe insertion (Burg et al., 1995). It was also reported that the Qβ replicase preferred to replicate MDV-1 without probe insertions as opposed to those with probes (Lizardi et al., 1988). As to the negative control (no template reaction), the response time was about 26.2 min, which was close to that obtained for 10⁶ molecules of MDV-HLP and MDV-HSP. The size and integrity of each amplicon were checked by 2% agarose gel electrophoresis. As is shown in Figure 5.12, ~300 nt RNA was produced in the reaction without template added (negative control). In order to test if RNA could be produced in a negative control every time, only reactions of negative control were performed and the amplified product was tested on agarose gel. As is shown in Figure 5.13, for each negative control, unexpected RNA was produced.
Figure 5.11. RNAs amplified by Qβ replicase. The initial concentration of RNA in the reaction is $10^6$ molecules. Each point shown in the figure is the mean value of duplicate test.

Figure 5.12. Agarose gel electrophoresis of amplified RNAs by Qβ replicase. M: low range ssRNA ladder (Cat. No. N0364S, New England Biolabs); Lane 1: negative control; Lane 2 & 3: amplified MDV-HLP (334 nt); Lane 4 & 5: amplified MDV-HSP (305 nt); Lane 6: amplified MDV-poly (244 nt).
5.4. Discussion

Since the initiation of the research on nucleic acid hybridization technology in 1961 (Hall & Spiegelman, 1961), a number of formats for nucleic acid hybridization have been developed such as Southern blotting (Southern, 1975), Northern blotting (Alwine et al., 1979), in situ hybridization (Gall & Pardue, 1969; John et al., 1969), PCR (Mullis et al., 1986), and other isothermal amplifications including Qβ replicase reaction assay, NASBA (Guatelli et al., 1990), LAMP (Notomi et al., 2000), and helicase–dependent isothermal amplification (HDA) (Vincent et al., 2004). For safety and convenience, the hybridization probes incorporated in these methods have been developed from originally radioisotope-labeled probes such as $^{32}$P, $^{125}$I, $^{35}$S, and $^3$H labeled probes (John et al., 1969; Southern, 1975), to non-radioactively labeled probes such as biotin- (Broker et al., 1978; Chu et al., 1986) and digoxigenin-labeled probe (Kessler et al., 1990; Kessler, 1991), and later developed to fluorescent probes such as adjacent probes (Wittwer et al., 1997), Taqman probes (Livak et al., 1995), molecular beacon...
probes (Tyagi & Kramer, 1996), and strand-displacement probes (Li et al., 2002). All these nucleic acid hybridization methods rely on target amplification, except that only the Qβ replicase assay focuses on the amplification of the hybridized probe. The product generated during the Qβ replicase amplification reaction can be monitored in a real-time mode by testing the fluorescence of RNA-intercalated dyes. Propidium iodide was used in the early reports (Burg et al., 1995; Smith, et al., 1997a, 1997b; Stefano et al., 1997; Stone et al., 1996), and SYBR Green II is mostly used in recent years (Bansho et al., 2012). We compared the two dyes and found that SYBR Green II gave more sensitive signal than propidium iodide (data not shown). The “response time” used in the Qβ replicase assay is similar to the cycle threshold (Ct) value in real-time PCR. Another difference between PCR and Qβ replicase assay is that the latter assay contains an integrated target capture and purification step. Thus, RNA purification normally included in the steps prior to PCR amplification can be eliminated in the Qβ replicase assay. The chaotropic salt guanidine thiocyanate (An et al., 1995; Shah et al., 1995; Tyagi et al., 1996) incorporated in the hybridization buffer of the Qβ replicase assay lyses cells, releases viral RNA, and protects RNA from degradation by nucleases (Lomeli et al., 1989). Moreover, guanidine thiocyanate can lower the hybridization temperature (Thompson & Gillespie, 1987), which enables the process to be conducted at 37°C.

The first step in the Qβ replicase assay is designing target specific probes, including reporter probe and capture probe. The reporter probe is a MDV-1 based recombinant RNA. The specific target-complementary sequence needs to be inserted into a special position of MDV-1, in order that the native secondary structure of MDV-1 is not disturbed by the insert, which guarantees the new recombinant RNAs are able to be replicated by Qβ replicase (Lizardi et al.,
This position is located between the nucleotide 63 and 64 of MDV-1 (+) RNA (Miele et al., 1983). A polylinker containing BgIII, XbaI, XhoI, and Stul has been embedded in that position of the cDNA sequence of MDV-1 (+) enclosed in plasmid pUC-MDV-LR. These four restriction sites can be used for inserting target specific cDNA sequences. Generally, in order to insert a DNA fragment into a plasmid vector, it is preferable to use two different restriction enzymes to digest the plasmid so that different ends are generated, and this procedure prevents the religation of the vector itself, and also allows the fragment to be inserted in a directional manner (Davis et al., 1994). However, another XbaI site was discovered on the backbone of pUC-MDV-LR, XhoI was found with very low cutting efficiency in our experiment, and BgIII and Stul were not able to work effectively in the same buffer simultaneously. Under these conditions, BgIII was the only choice for the digestion of pUC-MDV-LR.

The HAV specific probe sequence in our experiment was chosen from the most conserved region of the viral genome located at 5’ end (~600 nt) (Jothikumar et al., 2005). Through the alignment of the 5’ end of 23 strains from different genotypes, the probe sequence was selected from the region between nucleotide 230 and 560, based on the sequence of HAV HM175/24A (GenBank accession number M59810.1). Different lengths of probe sequences were generated by the Array Designer 4.0 software (Premier Biosoft, Palo Alto, CA, USA). Then, the secondary structure of MDV-1 with different inserts was tested by the software DNAman 6.0 (Lynnon LLC., San Ramon, CA, USA). The probe sequence, UGCAAGGUGA CGUUCCAAACAUUCUGUGUCCCCAAUUUAGACUCUACAGCUCCAU, which perfectly retained the native secondary structure of MDV-1, was finally used in the current study. Burg et al. (1996) found that inserting a RNA probe into MDV-1 could greatly decrease
the amplification efficiency of Qβ replicase. They also reported that spacer elements flanking the target-specific probe could not only improve the replication rate of reporter probe but also enhance the hybridization efficiency of the probe to a target. In the current study, we designed two different reporter probes, one with spacer elements (MDV-HLP) and the other one without the elements (MDV-HSP). These two probes had the same HAV-complementary sequence and maintained the secondary structure of the MDV-1 domain. Both reporter probes could be replicated in our experiment. We found that the recombinant RNAs replicated at a lower rate than MDV-poly, and this is in good agreement with a previous report, suggesting that the presence of inserts might change the tertiary RNA confirmation of MDV-poly (Burg et al., 1995). Stone et al. (1996) also reported that the insertion of a heterologous sequence into MDV-1 could reduce the replication rate of RNA. Axelrod et al. (1991) found that recombinant RNAs exhibited replication rates from 25% to 69% of that of MDV-1. It was also reported that different reporter probes demonstrated different response times (Stone et al., 1996). In one published report, the response time of MDV-poly at the concentration of $10^5$ copies/reaction was 15 min (Lizardi et al., 1988). The replication rate of MDV-poly in our research was lower than the reported value, which might be caused by the different quality of Qβ replicase. Qβ replicase isolated using different methods has different replication quality (Gunasekaran et al., 2013; Kamen et al., 1972; Moody et al., 1994).

The capture probe, a short ssDNA sequence specific to targets, functions to lower the non-specifically bound reporter probes (Shah et al., 1994; Shah et al., 1995; Shah et al., 1995; Tyagi et al., 1996). The sequence of capture probe was also selected from the 5’ end of the HAV genome. Two capture probes were designed in the current study. The first capture probe
functions to fix the hybrid of target and reporter probe on a solid support in order to remove the excess non-hybridized reporter probes by thorough washing. However, this extensive washing cannot ensure that all non-hybridized reporter probes are removed (Lomeli et al., 1989). Even a single copy of non-hybridized reporter probe left in the reaction could generate detectable fluorescent signal, obscuring the presence of rare targets (Kramer & Lizardi, 1989).

Several methods have been developed by two research groups to eliminate the residual non-hybridized reporter probes and reduce background signal. The first method developed by Gene-Trak Corporation was named “reversible target capture” (RTC). In this approach, two capture probes bearing different affinity ligands were utilized in the hybridization procedure to reduce carry-over of reporter probes. One capture probe had a tail of 150 deoxyguanosine residues and the other one had a tail of 150 deoxyadenosine residues (An et al., 1995; Shah et al., 1995a, 1995b). Alternatively, the first one was biotin labeled and the second one had a 150 deoxyadenosine tail (Shah et al., 1994). This approach suffered from the large number of assay steps and was very time-consuming. Blok & Kramer (1997) tried to insert a molecular switch into the reporter probe that did not interfere with the replication of the probes. The molecular switch was a complementary 21-nucleotide sequence that could be recognized by *E. coli* ribonuclease III (RNase III). This switch could change conformation upon hybridization of reporter probe to its target, protecting the hybridized probes from attack by RNase III and leaving non-hybridized reporter probes destroyed by the enzyme. However, the researchers did not obtain the expected results and found that all reporter probes were attacked by the RNase III due to the fact that the hairpin stem of the molecular switch was too long and the conformational change did not occur during hybridization (Blok & Kramer, 1997). This research group also developed another approach by using two reporter probe fragments to
hybridize to adjacent positions on the HIV genome. After hybridization, the two probe parts were ligated with T4 DNA ligase to become a whole reporter probe. The hybrid of reporter probe:target was released by RNase H that specifically cleaved RNA hybridizing to DNA (Crooke et al., 1995; Inoue et al., 1987; Nakamura et al., 1991; Stein & Hausen, 1969). The method performed well but the overall efficiency was limited by the ligation step (Tyagi et al., 1996). In order to improve the efficiency, we modified this method by utilizing the second capture probe to hybridize to non-hybridized reporter probes and then employed RNase H to digest RNA that hybridized to DNA, thus breaking the non-hybridized reporter probes into two parts. In order to protect hybridized probes from being attacked by RNase H, we needed to ensure the extreme specificity of capture probes. The sequence alignment analyses of capture probes and reporter probes revealed that the first capture probe could not hybridize to the reporter probe and the second capture probe only hybridized to the designated position of the reporter probe. The stable secondary structure of MDV-1-based reporter probes could also block the cleavage of RNA by RNase H (Donis-Keller, 1979).

RNAs were detected in template-free reactions in the current study, which was also reported by other researchers (Biebricher, 1981a, 1981b; Hill & Blumenthal, 1983; Nakaishi et al., 2002; Sumper & Luce, 1975). The RNA synthesis became detectable after a lag phase of 20 – 40 min of reaction (Hill & Blumenthal, 1983; Sumper & Luce, 1975), which was similar to our result that the fluorescent signal in the template-free reaction was detectable at around 26 min. The fluorescence signal in the no template reaction was close to that observed for $10^6$ molecules of template in the current study. Nakaishi et al. (2002) found that the detectable signal of no template reaction was close to that for $10^{10}$ copies of template. The mechanism of
RNA synthesis in template-free reactions continued to be an area of active interest and debate. Some researchers insisted that the Qβ replicase purified in their lab was free of contaminating RNAs and ascribed the origin of self-replicating RNAs to an ability of the replicase to synthesize RNAs de novo in the presence of nucleoside triphosphates in the reaction followed by the selection of the faster replicating RNA species (Biebricher et al., 1981a, 1981b; Biebricher et al., 1986; Biebricher et al., 1993; Sumper & Luce, 1975). However, Hill & Blumenthal (1983) stated that spontaneously synthesized RNAs arose from the contamination of RNAs in E. coli cells. They employed a urea-phosphocellulose column to purify Qβ replicase and obtained highly purified enzyme incapable of spontaneous RNA replication. However, Biebricher et al. (1986) repeated the Qβ replicase purification methods of Hill and Blumenthal and found that spontaneous RNA synthesis did occur. Chetverin et al. (1991) claimed that the origin of RNAs was from the laboratory environment. Moody and co-workers (1994) extracted Qβ replicase from E. coli cells transformed with a plasmid containing the gene encoding the β subunit of Qβ replicase under aseptic conditions and the researchers could totally suppress the RNA synthesis in template-free reactions or delayed the detectable signal to later than 45 min. These researchers also sequenced the spontaneous replication RNAs and found that these RNAs were related to E. coli tRNA. Based on the above findings, it was concluded that spontaneous production of RNAs in template-free reactions was derived from the evolution and amplification of low levels of E. coli RNA contaminating the enzyme preparation (Moody et al., 1994). The researchers disagreed with the idea that the RNAs arose by the de novo assembly of nucleotides by Qβ replicase. In order to know what was produced in the template-free reaction in our experiment, we analyzed the RNA products of no template reactions conducted independently through agarose gel electrophoresis and found distinct
banding patterns on gel. Bebricher and coworkers (1981 a & b) also reported that different lengths and species of RNAs were produced by the same Qβ replicase in independent template-free reactions (Biebricher et al., 1981a, 1981b). Chetverin et al. (1991) reported non-reproducible band patterns of template-free reactions as well.

However, for the non-template reaction control, either no RNA synthesis (Burg et al., 1995; Hill & Blumenthal, 1983; Tyagi et al., 1996) or low levels of RNA synthesis (Shah et al., 1994; Shah et al., 1995a, 1995b) were reported. In contrast to our results where the fluorescent signals in all no-template controls were detected relatively early. The reason for these contradictory findings maybe attributed to different Qβ replicases used in each study. Qβ replicase can be purified from *E. coli* cells infected with Qβ phage (Biebricher et al., 1981a, 1981b; Biebricher et al., 1986; Kamen et al., 1972; Sumper & Luce, 1975), from *E. coli* cells containing the plasmids carrying the gene of β subunit of Qβ replicase (Burg et al., 1995; Moody et al., 1994), or from *E. coli* cells co-transformed with the plasmids containing the genes encoding subunit α, β, γ, and δ, respectively (Gunasekaran et al., 2013). Different sources of enzyme gave different sensitivity and background signal. The enzyme used in our research was from Epicentre Biotechnologies. The description of Qβ replicase in the statement of the product was “Qβ replicase incubated in the presence of rNTPs with no template present must not generate RNA transcripts by gel analysis”. However, we obtained the opposite results to the description. We could only buy Qβ replicase from Epicentre Biotechnologies, the only company selling the enzyme. After communicating with them about our findings, Epicentre Biotechnologies discontinued their Qβ replicase.
The research on Qβ replicase has been a hot area for many years. Several labs, especially the research group of Dr. Chetverin in the Institute of Protein Research, Russian Academy of Sciences, and the research group of Dr. Yomo at Osaka University, Japan, have been studying the properties of Qβ replicase for decades. Currently, the high background signal in the template-free reactions makes the Qβ replicase assay inappropriate for the detection of HAV in food. However, if in future, RNA synthesis in no template reaction is eliminated, the Qβ replicase reaction assay will be a promising method for pathogen detection in food.

5.5. Conclusions

The Qβ replicase reaction assay was employed to detect the genomic RNA of hepatitis A virus. Two MDV-1 based reporter probes, MDV-HLP and MDV-HSP, were synthesized. Both probes were complementary to a conserved region of HAV genome. These two probes could be amplified by Qβ replicase, with MDV-HLP giving a faster replication rate than MDV-HSP. The critical step of Qβ replicase reaction assay is removing non-hybridized reporter probes, which could cause false positive results. A system for elimination of non-hybridized reporter probes was proposed in our report. RNA synthesis was found in template-free reactions and the time to obtain a detectable fluorescent signal was similar to that for an assay containing $10^6$ molecules of templates. This makes the assay not applicable for the detection of HAV in food currently. In future, the Qβ replicase reaction assay will be a promising isothermal amplification technique in foodborne virus detection, only if the RNA synthesized in the no template control is completely stopped.
Chapter 6: GENERAL CONCLUSIONS AND FUTURE RESEARCH

6.1. General conclusions

In the current study, we developed a method based on iron oxide magnetic nanoparticles for the concentration of HAV from different food matrices, and also used isothermal amplification technologies, RT-LAMP-BART and Qβ replicase reaction assay, for HAV detection. The major findings of the thesis are summarized as follows:

(1) We successfully coated the surface of Fe₃O₄ MNPs with -NH₂ groups, -COOH groups, and protamine. The MNPs were coated with -NH₂ groups using four different treatments including shaking in water at 50°C for 24 h, shaking in ethanol (90%, v/v) at 50°C for 24 h, sonicating in water for 10 h, and sonicating in ethanol (90%, v/v) for 10 h. Zeta potential analysis indicated that shaking MNPs in water resulted in MNPs with the highest level of -NH₂ groups on the surface. The pI of MNPs shifted firstly to basic pH after coating with -NH₂ groups, shifted to acidic pH after coating with -COOH groups, and then shifted back to basic pH after coating with protamine, indicating the successful surface modification of MNPs. Zeta potential tests showed that both NMNPs and PMNPs were positively charged at pH lower than 10. FTIR and TEM techniques were used to verify the successful surface modifications of MNPs. The specific bands of the corresponding chemical groups and a thin layer of protamine coating were observed in the FTIR spectra and TEM micrographs of the modified MNPs, respectively.
(2) We investigated the feasibility of PMNPs and NMNPs for recovering HAV from viral suspension (50 mL) at different pH, NaCl concentration, and MNP concentration, and found the optimal condition was pH 9.0, 0.14 M NaCl, and 50 µL of MNPs. The recovery rate obtained under these optimal conditions was between 24% and 49% depending on HAV inoculation level.

(3) We compared the efficiency of three RNA extraction methods, namely HCl and Kit method, Kit method, and Heating method, in using MNPs to concentrate HAV from viral solution, and found that the Kit method was the best for extracting RNA from HAV captured by PMNPs while the HCl and Kit method worked most efficiently for the RNA extraction from HAV recovered by NMNPs. NMNPs and PMNPs were then employed to concentrate HAV from green onions (15 g), strawberries (50 g), mussels (5 g), and milk (40 mL) artificially contaminated with HAV. Glycine buffer (pH 9.0) containing 0.05 M glycine, 0.2% (v/v) Tween 20, and 0.14 M NaCl was capable of efficiently washing HAV particles from green onions, strawberries, and mussels. After HAV was eluted from strawberries, centrifuging the eluate to remove food debris form the HAV suspension was found to be essential, because otherwise HAV RNA could not be detected in the suspension. However, this centrifugation treatment was found to be not necessary for processing HAV-bearing eluate from green onions. PMNPs demonstrated higher consistency than NMNPs in concentrating HAV from green onions, strawberries, mussels, and milk, indicating that, aside from electrostatic attraction, other forces such as hydrogen bonding and hydrophobic interactions between protamine and the virus capsid might be responsible for the excellent HAV capturing ability of PMNPs. Using real-time RT-PCR, the limit of detection of HAV concentrated by
PMNPs from green onions, strawberries, mussels, and milk were 8.3 PFU, 83 PFU, 8.3 PFU, and 8.3 PFU, respectively.

(4) We also used a PEG dialysis method to capture HAV from green onions, strawberries, mussels, and milk, and compared this method with MNP capture method. The dialysis method demonstrated a similar limit of detection of HAV from green onions, mussels, and milk to that observed for the PMNPs method. However, the PEG dialysis method took longer time than the PMNPs method (8 h vs. 50 min) but is less labor intensive than PMNP method.

(5) RT-LAMP-BART technique was used for the first time in foodborne HAV detection in the current study. A one-step RT-LAMP-BART (RNA-LAMP-BART) assay performed at 55°C and a two-step RT-LAMP-BART (cDNA-LAMP-BART) assay performed at 62°C were employed to detect HAV concentrated from green onions, strawberries, mussels, and milk. Magnesium ion concentration was optimized to improve the sensitivity of RNA-LAMP-BART and cDNA-LAMP-BART. The optimal Mg$^{2+}$ concentration for RNA-LAMP-BART and cDNA-LAMP-BART was found to be 2 mM and 4 mM, respectively. In addition to adjust the concentration of the reaction components, the sensitivity of RT-LAMP-BART could be enhanced by using primers with higher purity. It was found that HPLC-purified primers demonstrated higher sensitivity than cartridge-purified primers in amplifying purified HAV RNA. The cDNA-LAMP-BART demonstrated a limit of detection of 8.3, 83, 8.3, and 8.3 PFU for HAV in green onions, strawberries, mussels, and milk, respectively, and the results were comparable to those of a two-step real-time RT-PCR. Due to the isothermal amplification
characteristic and simplicity of the light signal detection, the instrument for LAMP-BART
assay has the potential to be made portable to satisfy field applications.

(6) Qβ replicase reaction assay, an isothermal amplification technology based on nucleic acid
hybridization and Qβ replicase amplification, was used to detect HAV in the current study.
Two MDV-1 based reporter probes, containing HAV specific complementary sequences, were
generated by transcription with T7 RNA polymerase from recombinant plasmids. The two
reporter probes retained the native secondary structure of MDV-1 and were able to be
recognized and amplified by Qβ replicase. Two HAV specific capture probes were chosen
from the 5’-UTR of HAV genome. A problem associated with the Qβ replicase reaction assay
is the strong background signal caused by the residual non-hybridized reporter probes. An
experimental protocol was designed to remove non-hybridized reporter probes using RNase H
that only digested RNA hybridizing to DNA. However, we found that the reaction without
template could generate signals (false positive result), and this finding was in good agreement
with previous reports (Biebricher, 1981a, 1981b; Hill & Blumenthal, 1983; Nakaishi et al.,
2002; Sumper & Luce, 1975). Future research efforts should be focused on developing RNA-
free Qβ replicase. When such high-purity Qβ replicase is available in future, the Qβ replicase
reaction method will see great application in foodborne pathogen detection.

6.2. Future research

Findings in the current study can provide useful information and suggestions for future studies
in the area of concentration and detection of foodborne pathogens.
6.2.1. Concentration of different contaminants from food using charged MNPs

PMNPs showed good performance in concentrating HAV from different food eluates in our study. Although electrostatic attraction force-based method is less specific compared with antibody capturing method, antibody-based methods are too specific to capture all possible strains of a target pathogen. Moreover, the electrostatic attraction force-based method can be used to simultaneously capture different pathogens from food samples. Aside from HAV, PMNP will be a good tool for concentrating other contaminants in food. From the information shown in Table 1.3, most foodborne viruses are negatively charged at pH 9.0. Bacterial cell wall is also negatively charged. For Gram-positive bacterial pathogens, such as L. monocytogenes, C. perfringens, B. cereus, S. aureus, and C. botulinum, teichoic acids are present on the surface of cell wall, making cells negatively charged (Brown et al., 2013). For Gram-negative bacteria, such as Salmonella, E. coli, and Campylobacter, lipopolysaccharides cover the outer surface of cells, imparting a strong negative charge to bacterial cells (Dickson & 1988; Leone et al., 2007). Different pathogens might exist in the same food. Therefore, in future studies, the efficiency of PMNPs in concentrating several pathogens from the same food should be evaluated. As is shown in Table 1.1, aside from pathogens, there are also chemical and toxic contaminants in foods. PMNPs can also be investigated in removing chemicals such as heavy metal anions including AuCl₄⁻, PdCl₄²⁻, and HCrO₄⁻, as well as charged toxins, such as ricin with pI between 7.0 and 8.4 (Na et al., 2011) and neurotoxin of C. botulinum Type A with a pI of 6.1 (Dasgupta et al., 1970), from food or water. We also produced negatively charged COOH-coated MNPs that can be used in capturing positively charged contaminants such as hazardous heavy metal cations including Hg²⁺, Pb²⁺, Co²⁺, Mn²⁺, Cu²⁺, and Zn²⁺, as well as toxins such as ricin and neurotoxin of C. botulinum Type A, in food or water.
6.2.2. Application of surface modified Fe₃O₄ MNPs for capturing pathogens in food

We developed the protocol for surface modification of Fe₃O₄ MNPs. This developed protocol of step-by-step surface modification of MNPs with protamine is meaningful, because this method can also be used to attach other proteins or polymers to MNPs for the purpose of recovery of pathogens. Actually, using this method, we successfully attached porcine gastric mucin (PGM) to the surface of MNPs. It was reported that the PGM contained cell receptor of human NoVs (Tian et al., 2008). In our study, the FTIR spectrum of PGM-coated MNPs demonstrated specific bands indicating the successful coating of PGM (Figure 6.1). NoV is a prevalent pathogen frequently reported in foodborne outbreaks. The resultant PGM-coated MNPs will be used to capture NoVs from foods in future to improve the concentration efficiency of the virus.

![Figure 6.1. FTIR spectra of Fe₃O₄ MNPs uncoated and coated with PGM.](image)
This protocol of step-by-step surface modification of MNPs, developed in the current study, could also be used to attach other polymers to the surface of MNPs. Chitosan, produced by deacetylation of chitin, is an abundant natural aminopolysaccharide with biodegradability and non-toxicity and has been used to remove heavy metals from wastewater (Dutta et al., 2004). Using our developed protocol, chitosan can be coated onto the surface of MNPs, and chitosan-coated MNPs can be used to recover pathogens from foods in future.

6.2.3. Development of an easy-to-use apparatus to enhance the speed of PEG dialysis

In the current study, the PEG dialysis method demonstrated good performance and especially showed a high virus recovery rate from green onion. However, the processing time used in this current study was too long to satisfy the need of rapid detection. In future, a dynamic dialysis method should be developed to reduce the processing time. For example, an apparatus could be designed to speed up the circulation of PEG.

6.2.4. Further optimization of RT-LAMP-BART to improve the efficiency

The amplification time of RT-LAMP-BART used in the current study for the detection of HAV in foods was 130 min and was too long considering 40 min used for real-time RT-PCR. In future, the factors affecting the efficiency of RT-LAMP-BART need to be further optimized to increase the reaction speed. The Bst DNA polymerase with higher enzymatic activity needs to be developed, and the components of the reaction buffer need to be further optimized. Because of the simplicity of the instrument used in RT-LAMP-BART, a miniature device, such as a gene chip, could be designed for ease-of-use and rapid on-site detection in future.
6.2.5. Evaluation of the effect of exogenous RNA on the sensitivity of RT-LAMP-BART

In the current study, we only investigated the efficiency of RT-LAMP-BART in detecting HAV in different food matrices. Actually, food may be contaminated with more than one pathogen simultaneously. Thus, when testing HAV in food, the genome of non-target pathogens might be co-purified with HAV genome. The effect of the exogenous RNA on the sensitivity of RT-LAMP-BART needs to be tested in future.
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