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Development of a Novel Carbohydrate-Based Method for the Detection of Norovirus from Ready-To-Eat Foods.

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Department of Biochemistry, Microbiology and Immunology
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Abstract

Norovirus is an enteric pathogen transmitted via the fecal-oral route. It is a leading cause of outbreaks and sporadic cases of gastroenteritis worldwide. Outbreaks of norovirus are often linked to contaminated food or water. Ready-to-eat foods are particularly prone to contamination, since they often require handling during preparation and are not usually heated before serving.

Norovirus capsids have been shown to interact specifically with histo-blood group antigens (HBGAs). HBGAs are carbohydrates present on the surface of red blood cells, on mucosal epithelial cells and in bodily fluids. The work in this thesis used the interaction between norovirus and HBGAs to develop a method to detect norovirus in food samples.

Magnetic beads were coated with multiple types of HBGA [A, B, H(type 2) and H(type 3)]. These beads were added to a 250 mL volume sample and concentrated using either the Pathatrix™ or *i*CropTheBug. Both systems were able to concentrate the beads from a 250 mL sample to 140 µL, using the magnetic properties of the beads. The RNA was then extracted from the sample and the presence of norovirus was determined using realtime Reverse Transcription-PCR.

This method was successful at detecting norovirus from artificially-spiked and naturally-contaminated food samples. It was also able to detect multiple strains from norovirus genogroup I and II within the infectious dose range (10-100 particles). These results demonstrate that carbohydrate-conjugated beads can be successfully used to detect norovirus contamination of food products. This method can provide rapid and effective food testing for use in the investigation of suspected norovirus outbreaks.

Acknowledgments

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Table of Contents

Abstract ii

Acknowledgments iii

Table of Contents iv

List of Abbreviations vi

List of Figures viii

List of Tables ix

1 Introduction 1
1.1 Foodborne Disease 1
1.2 Norovirus 4
1.3 Norovirus Structure 5
1.4 Norovirus Classification 9
1.5 Histo-Blood Group Antigens 11
1.6 Histo-Blood Group Antigens and Norovirus 14
1.7 Norovirus Transmission 18
1.8 Norovirus Detection Methods 22
1.9 Hypothesis and Objectives 24

2 Materials and Methods 25
2.1 Norovirus Specimens 25
2.2 Feline Calicivirus 25
2.3 Histo-Blood Group Antigen Carbohydrates 25
2.4 Food Samples 28
2.5 Buffers 28
2.6 Magnetic Capture of Norovirus 30
2.6.1 Preliminary Binding Experiments 30
2.6.1.1 Direct Capture 30
2.6.1.2 Indirect Capture 30
2.6.2 Method Validation 31
2.6.2.1 Carbohydrate-Conjugated Magnetic Beads 31
2.6.2.2 *i*CropTheBug 31
2.6.2.3 Pathatrix™ 32
2.7 RNA Extraction 32
2.8 Norovirus Detection 33
2.8.1 Conventional RT-PCR 33
2.8.2 Realtime RT-PCR 35
2.8.2.1 Genogroup I Norovirus Assay 35
2.8.2.2 Genogroup II Norovirus Assay 35
2.8.2.3 Feline Calicivirus Assay 36
2.9 Standard Curve 36
2.10 Lectins 37
2.11 Absorption/Elution Method 38

2.12 Viral Typing 38

3 Results 40
3.1 Preliminary Binding Experiments 40
3.2 Optimizing Conditions for Norovirus Capture 42
3.2.1 Beads 42
3.2.2 Histo-Blood Group Antigens 44
3.2.3 Amount of Carbohydrates 45
3.3 Optimizing Large Volume Capture 50
3.3.1 Selection of Buffer 50
3.3.2 Direct Versus Indirect Capture 52
3.4 FCV as a Positive Control 54
3.5 Method Validation 57
3.5.1 Norovirus Detection in Buffer 57
3.5.2 Norovirus Detection from Food 59
3.5.3 Detection of Multiple Norovirus Strains 62
3.5.4 Detection of Naturally-Contaminated Samples 64

4 Discussion 66
4.1 Method Development 66
4.1.1 Internal Control 67
4.2 Method Validation 69
4.2.1 Detection from Buffer and Food Products 69
4.2.2 Pathatrix™ Versus *i*CropTheBug 71
4.3 Comparison to Other Methods 72
4.4 Conclusions and Future Work 74

5 References 75

List of Abbreviations

$3CL^{pro}$	3C-like protease
BDT	BigDye Terminator
CHO	Carbohydrate
cRNA	copy RNA
Ct	Critical threshold
dsDNA	Double stranded DNA
dsRNA	Double stranded RNA
ELISA	Enzyme-linked immunosorbent assay
FCV	Feline calicivirus
FITC	Fluorescein isothiocyanate
Fuc	Fucose
FUT	Fucosyltransferase
G	Genogroup
Gal	Galactose
GalNAc	N-Acetylgalactosamine
GlcNAc	N-Acetylglucosamine
HAA	*Helix aspersa*
HBGA	Histo-blood group antigens
IMS	Immunomagnetic separation
MNV	Murine norvirus
NTPase	Nucleoside triphosphate hydrolase
ORF	Open reading frame

PBS	Phosphate buffered saline
PFU	Plaque forming unit
RdRp	RNA dependent RNA polymerase
RFU	Relative fluorescence units
RHDV	Rabbit hemorrhagic disease virus
RT-PCR	Reverse transcription polymerase chain reaction
ssRNA	Single stranded RNA
UV	Ultraviolet
VLP	Virus like particle
VP	Viral protein
VPg	Viral protein genome-linked

List of Figures

Figure 1. Norovirus genome organization (Norwalk strain) 7

Figure 2. Structure of the Norwalk virus capsid and capsid protein 8

Figure 3. Phylogenic tree of the *Norovirus* genus 10

Figure 4. Biosynthesis of HBGAs 13

Figure 5. Model of norovirus – HBGA interaction 16

Figure 6. Binding of biotinylated carbohydrates to Dynabeads® 46

Figure 7. Binding of biotinylated carbohydrates to Matrix beads 49

List of Tables

Table 1. Characteristics of foodborne viruses 3

Table 2. Binding patterns of select norovirus strains or VLPs to human HBGAs 17

Table 3. List of selected norovirus outbreaks occurring from 2005-2009 21

Table 4. Source of norovirus strains 26

Table 5. Structure of biotinylated univalent and multivalent HBGA carbohydrates used for the capture of norovirus 27

Table 6. Composition of buffers used for the capture of norovirus from large volumes (250 mL) 29

Table 7. Primers and probes used for the detection of norovirus and FCV 34

Table 8. Preliminary binding experiments using magnetic beads to capture norovirus GII.4 2006b/Minerva 41

Table 9. Detection of 10-fold serial dilutions of norovirus GII.4 2006b/Minerva, using four different types of magnetic beads 43

Table 10. Capture of norovirus GII.4 2006b/Minerva using univalent HBGAs 45

Table 11. Capture of norovirus GII.4 2006b/Minerva using multivalent HBGAs 44

Table 12. Capture of norovirus GII.4 2006b/Minerva from 250 mL of various buffers 51

Table 13. Capture of norovirus GII.4 2006b/Minerva using direct and indirect binding 53

Table 14. Capture of FCV with sialic acid conjugated beads 55

Table 15. Detection of FCV used as a positive control from 25 g samples of lettuce using sialic acid or cationic beads 54

Table 16. Detection of norovirus GII.4, 2006b/Minerva in buffer using A-conjugated magnetic beads and A, B, H(2) and H(3)-conjugated magnetic beads with two automated extraction systems 58

Table 17. Detection of norovirus GII.4, 2006b/Minerva in buffer and food products using A, B, H(2) and H(3)-conjugated magnetic beads with two automated extraction systems 60

Table 18. Detection of multiple norovirus strains in buffer using A, B, H(2) and H(3)-conjugated magnetic beads with two automated extraction systems 63

Table 19. Detection of genogroup I and II norovirus from naturally-contaminated lettuce using both carbohydrate-conjugated magnetic beads and absorption/elution method 64

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1 Introduction

1.1 Foodborne Disease

Foodborne diseases are an important public health issue worldwide. In Canada there are an estimated 11 million cases of foodborne illness each year (141). These illnesses can be caused by a variety of microorganisms, toxins or chemicals. The economic impact of foodborne illnesses is considerable both in direct medical costs and indirect costs such as productivity and quality of life. Majowicz *et al.* (2006) estimated the annual cost of gastroenteritis illnesses in Canada was $3.7 billion (90). Despite the substantial number of cases and associated costs, the importance of foodborne diseases is often underestimated. This may be due to mild and under-reported illnesses. In addition, it is difficult to link cases directly to contaminated food products. Recently there have been several large foodborne outbreaks, *e.g.*, the Maple Leaf listeriosis outbreak, reported in the media. This has resulted in increased public awareness about foodborne illnesses and food safety issues (16, 153).

Numerous pathogenic bacteria, viruses or protozoan parasites can be transmitted via food products. The majority of individuals infected with these microorganisms experience mild gastroenteritis with symptoms of nausea, diarrhea and vomiting. Illness is normally self-limiting but can be more severe in children, the elderly and immuno-compromised individuals. Some foodborne pathogens such as *Clostridium botulinum*, *Listeria monocytogenes*, the hepatitis A virus and *Escherichia coli* O157 can cause serious neurologic, hepatic or renal disease. These infections are less frequent as compared to other enteric pathogens such as *Salmonella*. It is estimated that 5,000 people die each year in the United States as a result of foodborne illnesses (102).

Transmission of viruses through food products has been documented since the early 20th century (42). However, due to the limitations of detection methods and culturing techniques for foodborne viruses, research has focused on bacterial pathogens. It has only been in the past 20 years that rapid and reliable methods have become available for virus detection. The development of molecular methods has increased the diagnoses of illnesses linked to foodborne viruses. Currently, it is estimated that 67% of foodborne illnesses in the United States are caused by viral pathogens (102).

Several viruses are known to be transmitted through foods, including hepatitis A and E, norovirus, sapovirus, rotavirus, astrovirus, adenovirus and enterovirus (Table 1). These viruses are enteric viruses which grow in the human gastrointestinal tract, are excreted in feces and transmitted via the fecal-oral route. They are also non-enveloped, which allows them to persist in the environment. With the exception of rotavirus and adenovirus, they generally have a 30 nm icosahedral capsid and single-stranded RNA genome.

Enteric viruses present unique challenges for microbiological food safety. Unlike bacteria, they are stable in the environment and can persist for weeks to months on food products. Viruses are stabilized by the low temperatures used to inhibit bacterial growth, and can be preserved by freezing. The dose response of these viruses for humans is very low, with only a few particles required to cause illness (79, 140). Finally, since viruses cannot replicate outside of host cells, they are generally present at low levels on food products. Since there are very few viral culture systems available for enrichment, detection of viruses from foods is difficult.

Virus	Family	Genome	Envelope	Morphology	Size of virion (nm)	Size of genome (kb)	Culturable	Disease
Hepatitis A	Picornaviridae	(+) ssRNA	No	Icosahedral	27-32	7.5	Yes[1]	Hepatitis
Hepatitis E	Hepeviridae	(+) ssRNA	No	Icosahedral	32-34	7.2	No	Hepatitis
Norovirus	Caliciviridae	(+) ssRNA	No	Icosahedral	28-35	7.4-7.7	No	Gastroenteritis
Sapovirus	Caliciviridae	(+) ssRNA	No	Icosahedral	28-35	7.4-7.7	No	Gastroenteritis
Rotavirus	Reoviridae	dsRNA	No	Icosahedral	60-80	16-27	Yes	Gastroenteritis
Astrovirus	Astroviridae	(+) ssRNA	No	Icosahedral	28-30	7-8	Yes[1]	Gastroenteritis
Adenovirus	Adenoviridae	dsDNA	No	Icosahedral	70-90	28-45	Yes[1]	Respiratory, eye, and gastroenteritis infection
Enterovirus	Picornaviridae	(+) ssRNA	No	Icosahedral	28-30	7.2-8.4	Yes[1]	Poliomyelitis, meningitis and encephalitis

[1]Not all strains within the genus are culturable; wild-type strains are often difficult to culture

Table 1. Characteristics of foodborne viruses

Adapted from Greening, 2006 (46)

1.2 Norovirus

Outbreaks of norovirus have been documented since the 1920s (158). The illness was originally known as winter vomiting disease, due to the predominance of outbreaks in colder months. Many efforts were made over the years to identify the agent responsible for this disease. However, the virus was resistant to traditional cell culture methods. Norwalk virus was identified in 1972 by electron microscopic examination of stool samples from an outbreak at an elementary school in Norwalk, Ohio (71). Norwalk-like viruses with different antigenicity profiles were subsequently identified from similar outbreaks and these were subsequently grouped in the *Norovirus* genus (44).

Norovirus is a leading cause of sporadic cases and outbreaks of acute gastroenteritis worldwide. The illness is characterized by acute onset vomiting and diarrhea. Other symptoms may include low-grade fever, headache and nausea. Symptoms appear following a 24-48 hour incubation period and are self-limiting after 2-3 days (112). Despite the typically mild nature of the illness, severe side effects and fatalities have been reported (54, 146).

Norovirus research has been limited by the lack of a cell culture system or small animal model (36). As a result, limited knowledge is available on norovirus pathogenesis and host immune response. Recently, a few new methods for norovirus replication have been reported. A promising 3-dimensional cell culture system has been published for culturing norovirus (132). Unfortunately, efforts to replicate this system in other laboratories have failed. A method for replicating norovirus RNA in cell culture has also been reported, which may provide valuable insight into the molecular mechanism of norovirus replication (26). Additionally, gnotobiotic pigs and calves have been used successfully to replicate

human norovirus strains (130, 131). Finally, a culturable murine norovirus (MNV) has been identified, and is currently used by immunologists to characterize the immune response to norovirus (27, 72, 88). Previously, the immune response could only be studied with human volunteers. Despite these advances, a reliable method of replicating human norovirus in the lab is still needed.

Due to the public health importance of norovirus, a lot of research has focused on its environmental stability and resistance to disinfection. This is difficult to measure directly for norovirus without an infectivity assay. Most studies have used culturable viral surrogates, the feline calicivirus (FCV) and/or MNV, to estimate the behaviour of the human virus. The more recent murine system may be more relevant, as it is an enteric pathogen and resists more environmental conditions than FCV (18).

1.3 Norovirus Structure

Noroviruses are non-enveloped with a small (27 to 34 nm) icosahedral capsid, containing a single-stranded positive-sense RNA genome of approximately 7.5 to 7.7 kb. The genome consists of three open reading frames (ORFs); ORF 1 encodes a non-structural polyprotein, while ORF 2 and ORF 3 encode major and minor capsid proteins, respectively (Figure 1).

The non-structural polyprotein is processed by the viral 3C-like protease ($3CL^{pro}$) into six proteins. In order, from the N to C terminal, the proteins are: p48, NTPase, p22, VPg, $3CL^{pro}$ and RdRp. The functions of these non-structural proteins are not fully understood, due to the lack of a routine cell culture system for propagating human norovirus. However, the recent discovery of the culturable MNV could help advance the understanding of molecular mechanisms in norovirus (72). Proteins p48 and p22 are named based on their

respective molecular weight, although little is known about their functions (51). VPg is covalently linked to the norovirus genome and considered to be necessary for infection (15) Protease $3CL^{pro}$ is similar to the picornavirus protein 3C, and cleaves the polyprotein. The final protein in ORF 1 is the RNA dependent RNA polymerase (RdRp). Like most other RdRp, the norovirus RdRp lack proofreading ability, leading to increased genetic diversity and the development of quasispecies within infected individuals (22, 37). The RdRp region of the genome is fairly conserved within genogroups and has been the target of Reverse Transcription PCR (RT-PCR) primers for detection (2, 3).

The norovirus capsid is composed of 180 copies of the major capsid protein VP1 and one or two copies of the minor capsid protein VP2 (Figure 2) (116). VP1 and VP2 are synthesized from a subgenomic RNA containing ORF 2 and ORF 3 (51). The structure of the capsid has been solved by X-ray crystallography. VP1 forms a homodimer that self-assembles into a T=3 icosahedral virion (115). VP1 has two major domains, a shell domain (S) and a protruding domain (P), with the P domain subdivided into P1 and P2 sub-domains. P2 is a 127 amino acid residue hypervariable region within the P1 domain. In the tertiary structure of VP1, P2 forms the outermost surface of the protein, which protrudes from the assembled capsid. Residues in the P2 region are involved in histo-blood group antigens (HBGA) binding (138). Virus-like particles (VLPs) have been used to study norovirus particle structure. VLPs are structurally and antigenically similar to virus particles, except they lack RNA and are non-infectious (66). VP2 is not required for the assembly of VLPs, however, there is some evidence that it plays a role in particle stability (7). VP2 may also be involved in genome packaging, either through a protein-protein or protein-RNA interaction (51).

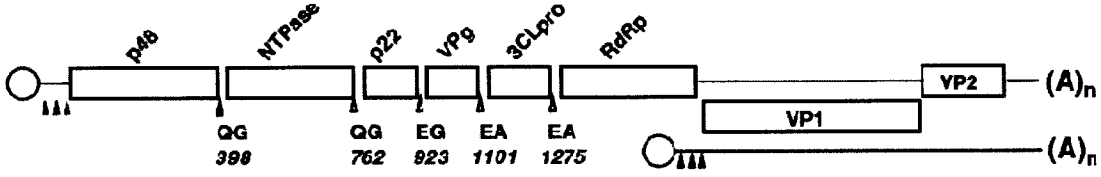

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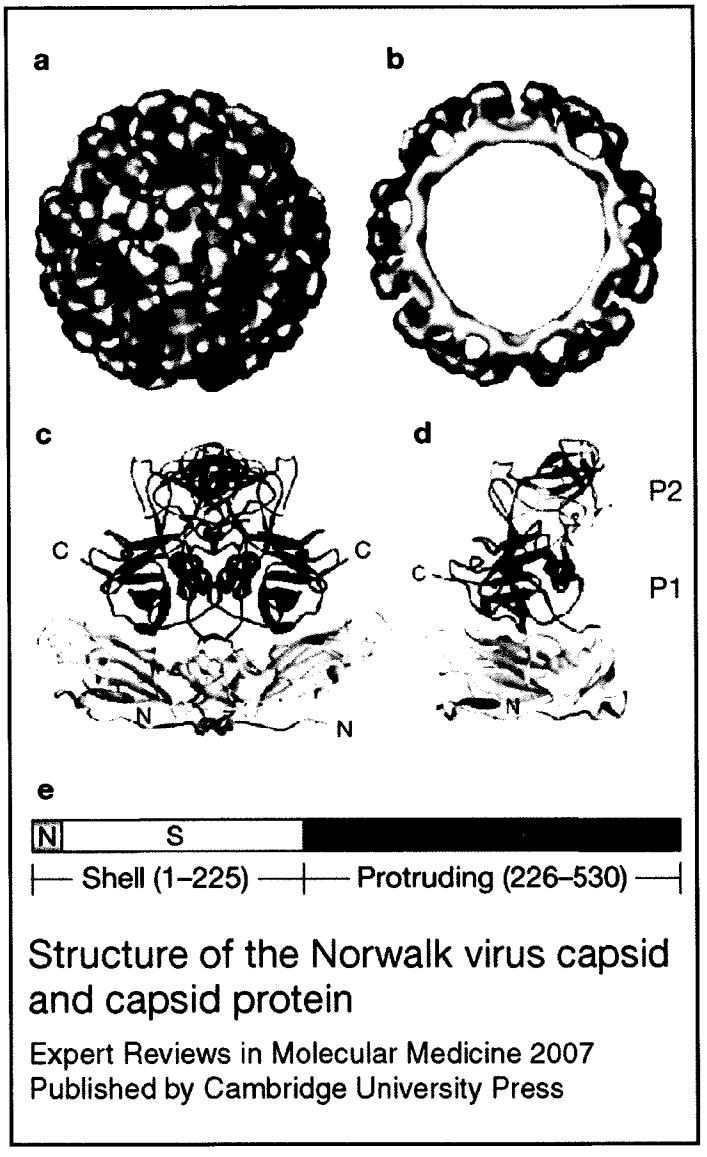
Figure 1. Norovirus genome organization (Norwalk strain)

Non-structural proteins in ORF1 are labelled and protease cleavage sites are indicated by open arrowheads. Amino acids numbers below the cleavage sites are the P1 residues of the recognition dipeptides. Filled arrowheads mark translation initiation codons. The VPg-linked subgenomic RNA encoding VP1 and VP2 is indicated below the ORFs. VPg is depicted as a circle linked to both genomic and subgenomic RNAs. Reprinted from Hardy, 2005 (51).



M. Tan and X. Jiang, Norovirus-host interaction: Implications for disease control and prevention, Expert Reviews in Molecular Medicine, Vol. 9, Issue 19, Pg. 1-22, 2007

Figure 2. Structure of the Norwalk virus capsid and capsid protein

(a,b) Surface and cross-sectional representations of the structure of recombinant Norwalk virus-like particle (VLP). (c) Dimers of capsid protein VP1, 90 dimers assembled in T=3 icosahedral symmetry to form the capsid. (d) Monomer of VP1 capsid protein, colour coded to show the different domains. The N-terminal arm region (green) faces the interior of the VLP, a shell (S) domain (yellow) forms the continuous surface of the VLP and a protruding (P) domain that builds up the arch-like structure emanating from the S domain surface. The P domain is divided into two subdomains – P1 and P2 (red and blue, respectively) – with the P2 at the outermost surface of the VLPs. (e) The linear representation of the capsid protein VP1, shown in the same colour theme. Reprinted from Tan and Jiang, 2007 (135).

1.4 Norovirus Classification

The *Caliciviridae* family contains four officially recognized genera: *Lagovirus*, *Vesivirus*, *Norovirus* and *Sapovirus* (45). Only noroviruses and sapoviruses are known to cause disease in humans and both produce acute gastroenteritis. The other viruses in the family cause a variety of diseases in animals, including rabbit hemorrhagic disease virus (RHDV) (*Lagovirus*) and FCV (*Vesivirus*). Recently, three new caliciviruses have been identified: bovine enteric calicivirus, tulane virus and st-valérien-like viruses, which have been proposed as three new genera of the *Caliciviridae* family (39, 86, 129).

The *Norovirus* genus is subdivided into genogroups, clusters (genotypes) and strains based on the similarity of the amino acid sequence of the major capsid protein VP1 (160). There are five genogroups (GI to GV) (Figure 3), and human norovirus strains are contained in GI, GII and GIV. Bovine and murine noroviruses make up GIII and GV, respectively. Animal norovirus strains have also been identified as belonging to GIV (feline and canine) and GII (porcine) (98, 99, 134, 151). Human-like strains have recently been detected in animal samples, suggesting the possibility of zoonotic transmission (101).

Strains belonging to either GI or GII are most commonly isolated from human cases. There are 14 and 20 current clusters (or genotypes) in GI and GII, respectively. One genotype, GII.4, is responsible for the majority of outbreaks. A retrospective study of worldwide norovirus outbreaks from 2001-2007, found that 62% were caused by GII.4 (126). The reasons for the predominance of GII.4 are not fully understood. Studies have shown that the GII.4 genotype is constantly evolving, and every few years a new dominant strain emerges. These new GII.4 strains evolve by antigenic drift, changing their surface properties to avoid herd immunity (17, 87, 125).

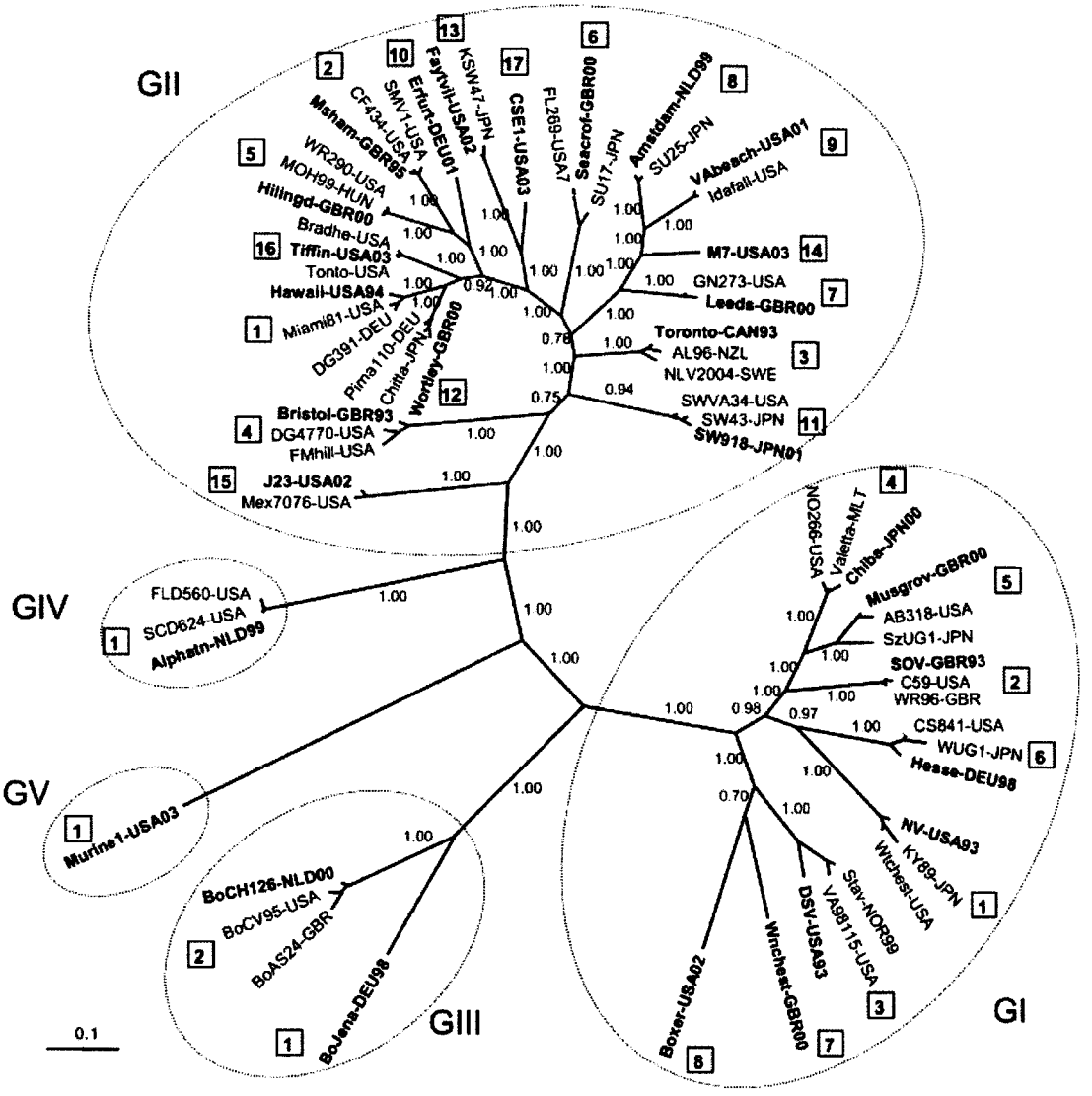


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Figure 3. Phylogenic tree of the *Norovirus* genus

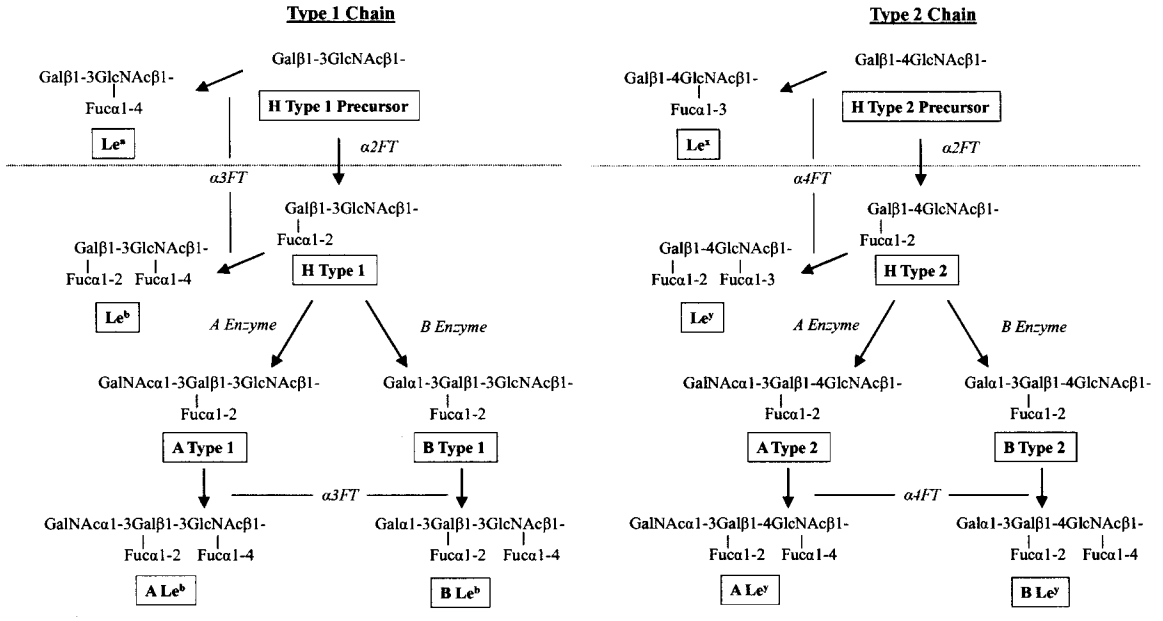
Genogroups (GI – GV) are represented by circles. Within each genogroup genotypes are indicated by square boxes. Reprinted from Zheng *et al*, 2006 (160)

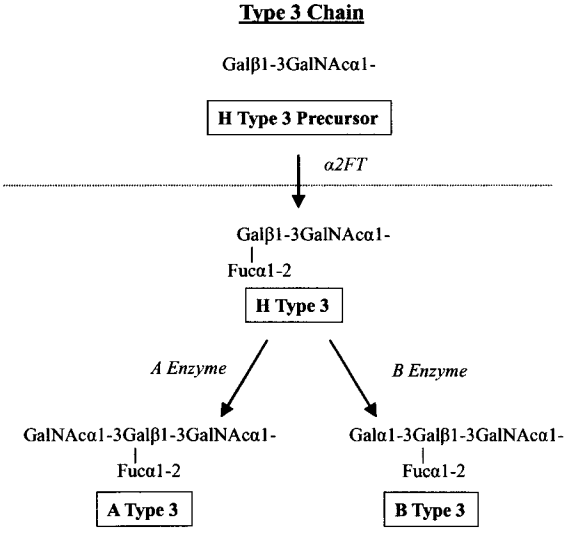
1.5 Histo-Blood Group Antigens

ABH and Lewis histo-blood group antigens (HBGAs) are complex carbohydrates located on the terminal end of cell surface carbohydrate chains. These HBGAs determine blood type. They are present on the surface of red blood cells, on mucosal epithelial cells and in bodily fluids (157). The interaction between norovirus and HBGAs has been well documented (see 1.6 Histo-Blood Group Antigens and Norovirus) (135, 136). HBGAs have also been shown to bind to bacteria (*Helicobacter pylori, E. coli* and *Campylobacter jejuni*) and other viruses (RHDV and Tulane Virus) (94, 138).

These antigens are synthesized from disaccharide precursor molecules with the stepwise addition of monosaccharides by glycosyltransferase enzymes (Figure 4) (94). There are three main precursor molecules, Type 1 (Galβ1-3GlcNAcβ1), Type 2 (Galβ1-4GlcNAcβ) and Type 3 (Galβ1-3GalNAcα1). The first step in the biosynthesis pathway of HBGAs is the addition of a α1,2 linked fucose molecule to the precursor molecule by α1,2fucosyltransferase (FUT1 or FUT2), to give H antigens. Approximately 20% of Europeans and 6% of Central Americans lack a functional FUT2 enzyme (14, 74). These individuals are considered non-secretors and do not express antigens in saliva or on certain epithelial cells. The second step in the pathway is the addition of α1,3 linked N-acetylgalactosamine (GalNAc) or a galactose (Gal) to the H antigens by enzyme A or B, respectively. It is the presence of enzyme A and/or B that determines the blood type, with individuals lacking both enzymes having blood type O (with the H antigen exposed on cell surfaces). The final step in the biosynthetic pathway is the addition of fucose (Fuc) by FUT3, a α1,3fucosyltransferase or α1,4fucosyltransferase, to GalNAc of the precursor molecule. In non-secretors, the addition of a Fuc to Type 1 precursor by α1,4 linkage

produces a Le^a antigen. Similarly, addition to a Type 2 precursor by α1,3 linkage gives a Le^x antigen. FUT3 can also catalyze the addition of a Fuc to a Type 1 A/B/O(H) antigen by a α1,4 linkage to give a A Le^b/B Le^b/Le^b antigen. Similarly, Type 2 A/B/O(H) antigens give A Le^y/ B Le^y/ Le^y antigens (94).


Type 1 Chain
Galβ1-3GlcNAcβ1-
Galβ1-3GlcNAcβ1-
Fucα1-4
H Type 1 Precursor
Le^a
α2FT
α3FT
Galβ1-3GlcNAcβ1-
Fucα1-2
Galβ1-3GlcNAcβ1-
Fucα1-2 Fucα1-4
H Type 1
Le^b
A Enzyme
B Enzyme
GalNAcα1-3Galβ1-3GlcNAcβ1-
Fucα1-2
Galα1-3Galβ1-3GlcNAcβ1-
Fucα1-2
A Type 1
B Type 1
α3FT
GalNAcα1-3Galβ1-3GlcNAcβ1-
Fucα1-2 Fucα1-4
Galα1-3Galβ1-3GlcNAcβ1-
Fucα1-2 Fucα1-4
A Le^b
B Le^b
Type 2 Chain
Galβ1-4GlcNAcβ1-
Galβ1-4GlcNAcβ1-
Fucα1-3
H Type 2 Precursor
Le^x
α2FT
α4FT
Galβ1-4GlcNAcβ1-
Fucα1-2
Galβ1-4GlcNAcβ1-
Fucα1-2 Fucα1-3
H Type 2
Le^y
A Enzyme
B Enzyme
GalNAcα1-3Galβ1-4GlcNAcβ1-
Fucα1-2
Galα1-3Galβ1-4GlcNAcβ1-
Fucα1-2
A Type 2
B Type 2
α4FT
GalNAcα1-3Galβ1-4GlcNAcβ1-
Fucα1-2 Fucα1-4
Galα1-3Galβ1-4GlcNAcβ1-
Fucα1-2 Fucα1-4
A Le^y
B Le^y


Type 3 Chain
Galβ1-3GalNAcα1-
H Type 3 Precursor
α2FT
Galβ1-3GalNAcα1-
Fucα1-2
H Type 3
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B Enzyme
GalNAcα1-3Galβ1-3GalNAcα1-
Fucα1-2
Galα1-3Galβ1-3GalNAcα1-
Fucα1-2
A Type 3
B Type 3

Figure 4. Biosynthesis of HBGAs

Three types of precursor disaccharides and their corresponding biosynthetic pathways are shown. Antigens are shown in boxes and enzymes required to catalyze each step depicted in italics. α2FT, α1,2fucosyltransferase; α3FT, α1,3fucosyltransferase; α4FT, α1,4fucosyltransferase. Individuals lacking a functional FUT2 enzyme, an α1,2fucosyltransferase, are non-secretors and will not express antigens below the dashed lines. Adapted from Marionneau *et al.* and Harrington *et al.* (52, 94).

1.6 Histo-Blood Group Antigens and Norovirus

ABH and Lewis HBGAs have been shown to bind norovirus capsids. This was first recognized by Marionneau *et al.* (2002) who demonstrated that Norwalk VLPs bind to epithelial cells from duodenal tissue sections in secretor individuals (95). The association had been hypothesized based on the interaction between a related calicivirus, RHDV and H Type 2 HBGA (118). Strains from other genera of *Caliciviridae*, specifically FCV and Tulane virus, also bind to carbohydrate ligands (133, 138). In addition, it has been recently reported that the bovine noroviruses (GIII) binds to an αGal epitope on HBGAs, which is present in bovids but not humans or swine (159). These finding raise interesting questions about the evolution of carbohydrate binding in the *Caliciviridae* family.

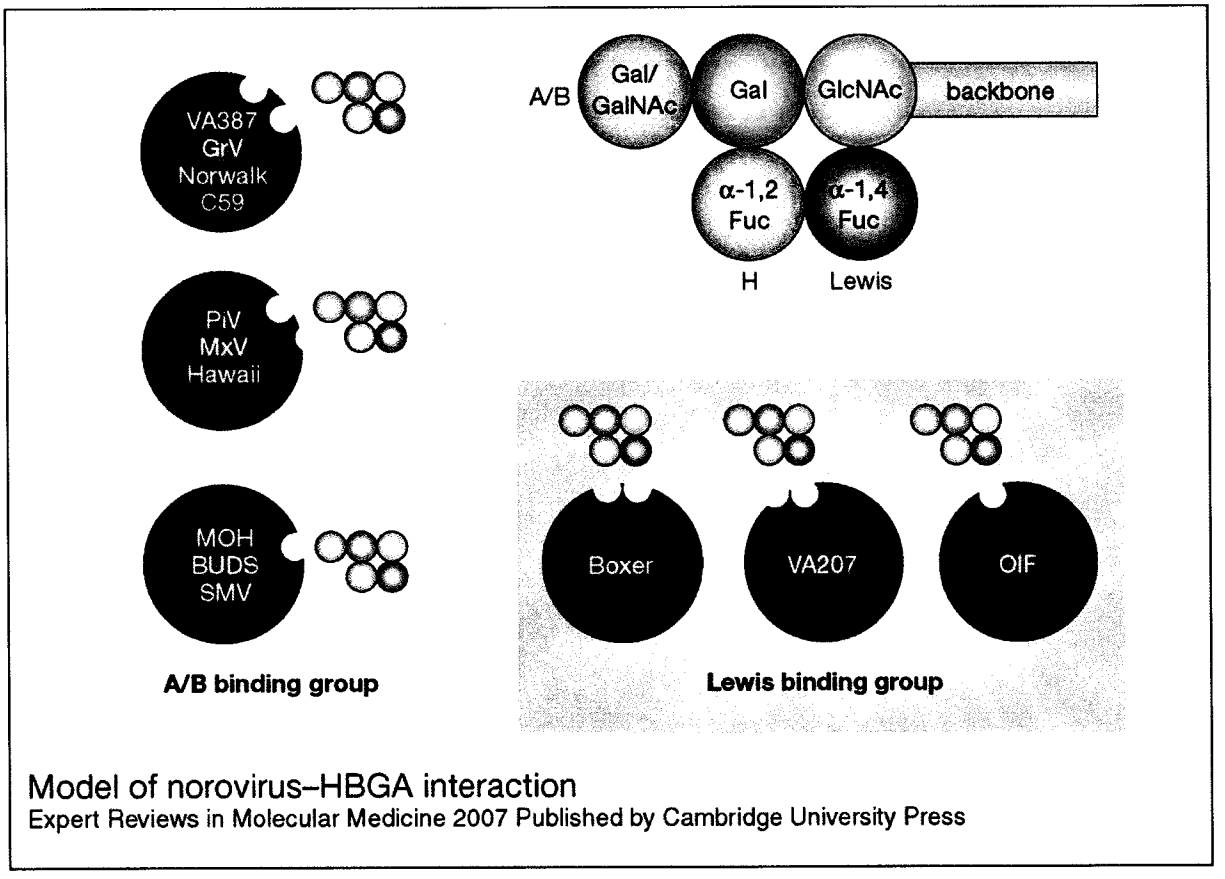
The binding specificities of several norovirus strains have been established using enzyme-linked immunosorbent assays (ELISAs) to measure the interaction between VLPs and saliva, synthetic oligosaccharides or glycosphingolipid HBGAs (53, 58, 107). The association between VLPs and HBGAs has been confirmed by experiments with infectious virus (53). Most noroviruses can bind multiple HBGAs (Table 2). Eight different binding patterns have been observed. They are divided into two groups: A/B binding and Lewis binding (58) (Figure 5). Also, the specificity of each strain for HBGAs is distinct and not necessarily shared between closely related strains.

The binding of norovirus and HBGAs is highly specific. Small changes in the structure of HBGAs can eliminate binding. X-ray crystallography and mutation studies have shown the importance of individual amino acids in the interaction between HBGA and the P dimer of norovirus (20, 29). Based on these studies, two distinct HBGA binding sites have been identified in GI and GII noroviruses. The binding sites are highly conserved within the

genogroup. The amino acids surrounding the binding sites are more variable and likely explain the different strain specificities for HBGAs (138).

The interaction between norovirus and HBGAs may explain the hypothesized genetic host factor involved in norovirus susceptibility. Observations from challenge studies and outbreaks have reported that some individuals, despite repeated exposure, never become infected and the attack rate in norovirus outbreaks rarely exceeds 70 %, despite the low infectious dose (108). Some studies have investigated the link between blood phenotype and norovirus susceptibility. A relationship between ABO blood type and susceptibility to norovirus strains has been reported by several studies (61, 137). A few studies have also been published refuting the link, however, they failed to consider the strain specific nature of blood type association (14, 48). In addition to blood type, secretor status has been clearly linked to susceptibility (60, 142). In general, non-secretors are not infected in norovirus outbreaks, although reports of a non-secretor infected in a Spanish nursing home outbreak and the presence of norovirus antibodies in non-secretors indicate that infection may occasionally occur in this population (21, 81). The factors involved in the susceptibility of non-secretors to norovirus are unclear.

Due to the reports linking HBGA phenotype and norovirus susceptibility, as well as the specific binding observed, it has been suggested that HBGAs are the receptor for norovirus. However, this is difficult to prove or disprove without a tissue culture system. In this thesis, the specific interaction between noroviruses and HBGAs was used to develop a sensitive and specific method for the extraction of viral particles from contaminated food products.



Model of norovirus–HBGA interaction
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Figure 5. Model of norovirus – HBGA interaction

This model is proposed based on the classification of two binding groups of norovirus to histo-blood group antigens (HBGAs): the A/B binding group and the Lewis binding group. The five-circle structure represents a pentasaccharide as the final product (ALe^b or BLe^b) of human HBGAs. The three major epitopes (A/B, H and Lewis) and the backbone are indicated. Thirteen norovirus strains are indicated according to their relative interaction levels with A/B, H and Lewis epitopes. The potential binding sites for each of the three major HBGA epitopes on the capsid are indicated. Reprinted from Tan and Jiang, 2007 (135).

Genotype	Strain	Carbohydrate binding								
		H1	H2	H3	Le^a	Le^b	Le^x	Le^y	A	B
GI.1	Norwalk	+	+	+	-	+	-	+	+	-
GI.3	Desert shield virus	-	-	-	-	-	-	-	ND	ND
GII.1	Hawaii	-	ND	-	-	+	ND	ND	+	+/-[1]
GII.2	Snow mountain virus	-	ND	-	-	-	ND	ND	-	+
GII.3	Toronto	+	ND	ND	ND	+	ND	ND	+	+
GII.3	Mexico	-	-	-	-	+	-	-	+	+
GII.4	Bristol	+	+	ND	-	+	-	-	+	+
GII.4	VA387	+	-	+	-	+	-	+	+	+
GII.5	MOH	+	ND	ND	-	+	-	ND	+	+
GII.9	VA207	-	ND	ND	-	-	+	+	-	-

+ Binding; - No binding; ND not done.
[1]Conflicting results.

Table 2. Binding patterns of select norovirus strains or VLPs to human HBGAs

Reprinted from Widdowson and Vinjé, 2008 (156)

1.7 Norovirus Transmission

Norovirus is transmitted via the fecal-oral route, either by person-to-person transmission or through contaminated food and water. A review of 226 outbreaks of acute gastroenteritis caused by norovirus in the US between 2000 and 2004 found that 35% of the outbreaks resulted from person-to-person transmission, 30% were foodborne, 5% waterborne and 30% had an unidentified source (10). Airborne transmission of the virus is also theoretically possible. Cases of norovirus transmission from aerosolized vomitus have been reported (96, 97). In addition, aerosolization of norovirus by flushing toilets has also been suggested as a possible mode of transmission (6).

Norovirus spreads easily from person-to-person, and a food or waterborne outbreak can be amplified by secondary cases. Several characteristics of norovirus are relevant for transmission: i) a low infectious dose, estimated to be as low as one infectious particle for Norwalk Virus (140) ii) high stability in the environment (5, 34) iii) resistance to many disinfectants (35) iv) high titres of norovirus shed in feces and vomitus (24, 25). Fomites and surfaces can be easily contaminated by vomiting events or poor personal hygiene. Because the virus is stable and resists disinfection, it can be difficult to control outbreaks when new susceptible populations are continually introduced. Norovirus outbreaks occur frequently in institutional and semi-closed settings such as nursing homes, hospitals, schools, hotels and cruise ships (28, 30, 33, 63, 70).

Outbreaks of acute gastroenteritis have been linked to norovirus contamination of drinking water (Table 3). These resulted from fecal contamination of drinking water sources and a failure of the water treatment system. They have been documented in small private water systems at resorts and restaurants, as well as in large municipal water systems (56,

109, 155). Interestingly, studies have detected norovirus in groundwater wells, tap water and mineral water in the absence of any reported outbreak (8, 12, 50). The virus was not confirmed to be infectious, but its presence raises water treatment and safety issues. Contaminated recreational water used for swimming has also been implicated in the transmission of norovirus (114, 120).

Food products may become contaminated with norovirus by contact with fecal matter during production, harvest, distribution or handling for preparation. Shellfish are the most common example of food contaminated during production, although contamination of produce from irrigation or wash water is also possible. Contaminated shellfish have caused several large, international norovirus outbreaks (82, 154). Shellfish are a high-risk food because they are filter feeders that concentrate microorganisms present in growing water and because there is a tendency to eat them raw or undercooked. Studies found relatively high levels of norovirus (100 - 1000 total viral particles) and multiple norovirus strains in shellfish (69, 84). Depuration, where shellfish filter clean water for a few days, is successful for eliminating bacterial pathogens. However, this has proved largely ineffective at removing viruses (123). This may be due to the presence of HBGA-like molecules in the shellfish which can bind noroviruses and enhance bioaccumulation (85).

The most common cause of foodborne norovirus outbreaks is contamination by infected food handlers (Table 3). Foods linked to these outbreaks include ready-to-eat items such as deli-meats, bakery products, fruits and vegetables (40, 83, 93, 122). Any product that requires handling (*e.g.*, chopping, mixing, peeling or icing) and is not subsequently heated before serving may transmit disease. Infected individuals shed high levels of virus in stool and shedding can persist for weeks after symptoms have resolved (4). It can be

difficult to pinpoint the source of an outbreak, due to problems detecting viruses in food products, where contamination may be at low levels and non-homogeneous.

Year	# of Cases	Country	Food/water type	Source or reason for contamination	Lab confirmed[1]	Strain	Reference
2009	299[2]	Italy	Municipal water	Failure of water treatment system	Yes	Not reported	(121)
2008	1699[2]	Montenegro	Municipal water	Failure of water treatment system	No	Multiple	(155)
2007	79	UK	Salads	Infected food handler	No	GII.6	(150)
2007	36	UK	Mixed salad	Sewage contamination	Not tested	Multiple	(124)
2007	30	Sweden	Oysters	Growth water	Yes	Multiple	(106)
2007	21	Austria	Ham roll	Food handler's infected child	Not tested	GII	(80)
2007	19	Australia	Oysters	Growth water	Not tested	Not reported	(59)
2007	38	Spain	Rice salad	Food handler	Not tested	Not reported	(31)
2006	218[2]	New Zealand	Drinking water	Sewage contamination	Yes	Multiple	(56)
2006	377	USA	Restaurant meals	Food handler	Not tested	GI.4	(11)
2006	111	Finland	Raw vegetables	Unknown	No	GII.1	(91)
2006	182	Austria	Salad	Food handler	Not tested	GII.7	(122)
2006	115	New Zealand	Oysters	Growth water	Yes	Multiple	(128)
2006	19	USA	Pot-luck meal	Unknown	Not tested	Multiple	(43)
2006	141[2]	USA	Well water	Sewage contamination	No	Multiple	(147)
2005	57	USA	Delicatessen meat	Food handler	Yes	GII.14	(93)
2005	87	USA	Lettuce	Food handler	Not tested	Single strain	(75)
2005	85	Japan	School lunch	Unknown	No	GII	(103)
2005	41	Italy	Raw mussels or ice	Unknown	Not tested	GII.3/GII.b	(117)
2005	1043	Denmark	Raspberries	During production	Not tested	Multiple	(38)

[1]Outbreaks were considered laboratory confirmed if norovirus was detected from the suspected source of the outbreak
[2] Individuals infected with other pathogens were included in the outbreak

Table 3. List of selected norovirus outbreaks occurring from 2005-2009

1.8 Norovirus Detection Methods

Due to the large number of norovirus outbreaks caused by contaminated food products; methods to detect noroviruses in foods are critical for epidemiological investigations. However, detecting viruses in foods is challenging and no standardized methods have been developed. Traditional food microbiology has focused on bacterial pathogens and these detection methods generally include an enrichment step to amplify the bacteria before plating on selective agar. Since viruses are unable to replicate outside of host cells and there is no reliable cell culture method for growing norovirus, it is not possible to increase the numbers of viruses extracted from food samples (36). Therefore, new and innovative methods need to be developed which are sensitive enough to detect the low numbers of particles present in foods.

Noroviruses have been classically identified by electron microscopy, a labour intensive procedure which requires high titres of particles (10^6/mL). ELISA assays have been developed to detect norovirus proteins, but they also require a high titre (10^5/mL) and cannot detect all norovirus strains (79). After the norovirus genome was cloned in the 1990s, genomic methods such as PCR were developed for detecting norovirus (64, 65). PCR-based methods can detect low numbers of virus and can be used with sequencing to give genotype information (145). They have improved the detection of norovirus from foods.

Some laboratories use commercial ELISA kits for diagnosing norovirus infections, however molecular detection is the established standard (79). Many RT-PCR primer sets have been developed, the most successful for detection and diagnostics target the conserved RdRp and ORF1/ORF2 junction (3, 73, 78). Realtime RT-PCR is more sensitive than conventional RT-PCR and the results obtained are semi-quantitative (145).

Since PCR-based methods require a small sample size and are sensitive to inhibition, steps are required to extract and concentrate viral particles from food samples prior to RNA purification. The complex and varied nature of food products presents a challenge for the development of extraction procedures. Methods previously used to concentrate norovirus include ultracentrifugation, polyethylene glycol precipitation, adsorption/elution filtration and immunomagnetic separation (IMS) (41, 47, 76, 77, 105, 111, 119).

Traditional IMS methods use specific antibodies conjugated to magnetic beads to separate particles of interest from the surrounding matrix. IMS has been used successfully for numerous applications including capturing bacteria from food products. Some IMS methods have been published for norovirus (77, 105, 111), However, it has not been possible to develop an antibody against all norovirus strains, limiting the effectiveness of these methods (49). Previous work in the lab used cationic (positively charged) beads to concentrate viruses from food samples. In theory, the cationic beads would attract the negatively charged capsids of all enteric viruses. This method worked very well for hepatitis A virus, but was not able to capture norovirus (110, 113).

1.9 Hypothesis and Objectives.

Based on specific binding between HBGA and norovirus, it was hypothesised that HBGA-coupled beads could be used to extract norovirus from food samples. The method proposed is similar to IMS, using carbohydrates in place of specific antibodies. A combination of HBGAs could be used to capture antigenically diverse strains.

The overall objective of this thesis was to develop a rapid and sensitive method to detect norovirus from foods. In order to maximize norovirus detection factors affecting norovirus capture were optimized in small volumes (1.5 μL). Experiments were done using different binding mechanisms (indirect and direct capture), different bead types, and carbohydrate structures as well as various amounts of carbohydrates to saturate the beads. After optimizing binding in small volumes condition affecting norovirus capture in large volumes (250 mL) were considered. These included different binding mechanisms (indirect and direct) and different buffers. Once the method was optimized it was tested for its ability to detect norovirus from various food products and ability to detect a range of norovirus strains.

2 Materials and Methods

2.1 Norovirus Specimens

Clinical stool specimens were obtained from acute gastroenteritis outbreaks in Canada from 2000-2007 (Table 4). Stool samples were diluted 1:5 (v/v) in phosphate buffered saline pH 7.2 (PBS) and mixed thoroughly. The solution was centrifuged for 20 min at 4000 × g, the resulting supernatant was filtered twice, first through a 0.45 μm Nylon membrane with graduated glass fibre filter (Millipore, Billerica, MA) and secondly through a 0.22 μm filter (Millipore). The filtrate was stored in 400 μL aliquots at -80°C until needed.

2.2 Feline Calicivirus

Seed cultures of Crandell's feline kidney cell line and FCV strain F9 were purchased from the American Type Culture Collection (Rockville, MD). Virus and cells were propagated as previously described by Bidawid *et al.* (9). Unconcentrated cell harvests containing FCV were pooled and stored in 1 mL aliquots at -80°C. The titre of FCV stocks was determined by plaque assay, as described previously by Bidawid *et al.*, to be 6×10^6 PFU/mL (9).

2.3 Histo-Blood Group Antigen Carbohydrates

Various biotinylated histo-blood group antigens were purchased from GlycoTech (Gaithersburg, MD) (Table 5). HBGAs were resuspended in 0.3 M sodium phosphate buffer (0.1 M NaH_2PO_4 and 0.2 M Na_2HPO_4, pH 7.4) to a final concentration of 1 mg/mL and stored at -20°C in 50 μL aliquots.

Strain	Source	Location	Date
GI.1	Oyster outbreak	British Columbia	2004
GI.3b	CDC stock[1]	United States	2007
GI.13	Cruise ship	Vancouver, BC	2004
GII.2	Cruise ship	Vancouver, BC	2004
GII.3	Catered event	Maple Ridge, BC	2004
GII.4 2002	Outbreak	Vancouver, BC	2004
GII.4 2004	CDC stock	United States	2007
GII.4 2006a	Family outbreak	Gatineau, QC	2007
GII.4 2006b	Catered event	Winnipeg, MB	2007

[1]Center for disease control (CDC)

Table 4. Source of norovirus strains

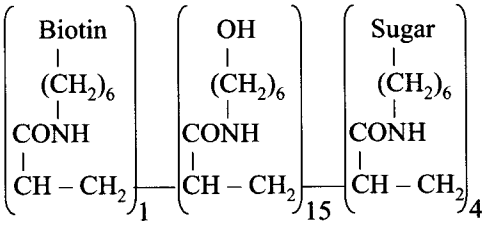
HBGA		Carbohydrate Moieties	Linker Chain
Univalent	Type A	GalNAcα1-3Galβ1- | Fucα1-2	Sugar – $O(CH_2)_3NHCO(CH_2)_5NH$ - Biotin
	Type B	Galα1-3Galβ1- | Fucα1-2	
	Type H	Fucα1-2Galβ1-	
	Lewis A	Galβ1-3GlcNAcβ1- | Fucα1-4	
	Lewis B	Galβ1-3GlcNAcβ1- | | Fucα1-2 Fucα1-4	
	Lewis X	Galβ1-4GlcNAcβ1- | Fucα1-3	
	Lewis Y	Galβ1-4GlcNAcβ1- | | Fucα1-2 Fucα1-3	
Multivalent	Type A	GalNAcα1-3Galβ1- | Fucα1-2	
	Type B	Galα1-3Galβ1- | Fucα1-2	
	Type H(2)	Galβ1-4GlcNAcβ1- | Fucα1-2	
	Type H(3)	Galß1-3GalNAca1- | Fuca1-2	
	Lewis A	Galβ1-3GlcNAcβ1- | Fucα1-4	
	Lewis B	Galβ1-3GlcNAcβ1- | | Fucα1-2 Fucα1-4	
	Lewis X	Galβ1-4GlcNAcβ1- | Fucα1-3	
	Lewis Y	Galβ1-4GlcNAcβ1- | | Fucα1-2 Fucα1-3	
	Sialic Acid	Neu5Acα2-6Galβ-	

Table 5. Structure of biotinylated univalent and multivalent HBGA carbohydrates used for the capture of norovirus

Univalent carbohydrates have a 1:1 ratio of carbohydrate moieties and biotin whereas multivalent carbohydrates have a 4:1 ratio of carbohydrate moieties and biotin. Sugar in the linker chain structure represents the location of the carbohydrate moieties.

2.4 Food Samples

Selected food products (lettuce, green onions, strawberries and deli-ham) were purchased locally and stored at 4°C until tested. For each experiment, the surface of 25 g food samples were sterilized under UV light for 30 min and inoculated with 50 µL of a norovirus filtrate diluted in PBS. A minimum of three 10-fold serial dilutions of the norovirus stock was tested for each experiment. The inoculum level varied from 1 to 10^7 copies due to natural variability in titre between samples. The virus inoculum was allowed to dry on the food sample for 30 min. Fifty µL of each virus dilution was also added to 90 µL of PBS in a microfuge tube to quantify the virus input for each experiment.

2.5 Buffers

Various buffers with pH values ranging from 3.0 to 9.0 were used for large volume (250 mL) capture of norovirus by HBGA-conjugated beads (Table 6).

Buffer	pH	Formula
Citrate	3.0	46.5 mM citric acid, 3.5 mM sodium citrate
Citrate	4.0	33 mM citric acid, 17 mM sodium citrate
Citrate	5.0	20.5 mM citric acid, 29.5 mM sodium citrate
Citrate	6.0	9.5 mM citric acid, 41.5 mM sodium citrate
Buffered peptone water	7.2	171 mM NaCl, 25 mM Na_2HPO_4, 11 mM KH_2PO_4, 1% peptone
PBS	7.2	137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4
Glycine	9.0	0.5 M glycine, 0.14 M NaCl, 0.2% tween 20

Table 6. Composition of buffers used for the capture of norovirus from large volumes (250 mL)

2.6 *Magnetic Capture of Norovirus*

2.6.1 Preliminary Binding Experiments

2.6.1.1 Direct Capture

Fifty μL of streptavidin-coated magnetic beads (Invitrogen, Carlsbad, CA) were dispensed into microfuge tubes. These beads were washed three times with 50 μL of PBS to remove preservatives, as per the manufacturers' instructions. Beads were mixed with 5 μg of biotinylated HBGAs for 30 min at room temperature on RKDynal rotary mixer (Invitrogen). The beads were washed three times with PBS to remove any excess carbohydrates and resuspended in 50 μL of PBS. HBGA conjugated beads were mixed for 30 min at room temperature with 50 μL of norovirus filtrate in a microfuge tube. The beads were then washed three times with PBS to remove any unbound virus.

2.6.1.2 Indirect Capture

Preliminary binding experiments were also done using indirect capture methods. Indirect binding is designed to give the interaction between the virus and HBGA more time and freedom. Fifty μL of norovirus filtrate was mixed with 5 μg of biotinylated HBGAs for 30 min at room temperature in a microfuge tube. Fifty μL of streptavidin-coated magnetic beads (Invitrogen) which were previously washed three times with PBS were added to the virus mixture. The samples were mixed for 30 min at room temperature on a RKDynal rotary mixer.

2.6.2 Method Validation

2.6.2.1 Carbohydrate-Conjugated Magnetic Beads

Fifty µL of M-270 streptavidin-coated magnetic beads were washed three times with 50 µL of PBS. The beads were mixed with 10 µg of multivalent biotinylated HBGA Type A, B, H(2) or H(3) for 30 min. The beads were washed three times with 50 µL PBS to remove excess carbohydrates, and stored at 4°C until needed. Streptavidin-coated magnetic beads (Matrix MicroScience Ltd., Newmarket, UK) were coated with 1 µg HBGA per 50 µL of beads following the same procedure, for use in the Pathatrix™ (Matrix MicroScience Ltd.). Cationic beads were also purchased from Matrix MicroScience for the capture of FCV as an internal control.

2.6.2.2 *i*CropTheBug

Inoculated food samples were transferred into a sterile Erlenmeyer flask containing 225 mL of citrate buffer pH 4. A total of 12.5 µL of each pre-coated A, B, H(2) and H(3) magnetic beads were added to the flasks. The flasks were mixed on an orbital shaker (Bel-Art Products, Pequannock, NJ) for 30 min. The beads were collected using a novel magnetic separation system, *i*CropTheBug (Filtaflex, Almonte ON, Canada). Briefly, the flasks were placed on the IMBCOL (immunomagnetic bead collector), a powerful settling magnet used to precipitate the magnetic beads. This was followed by 10 min on the IMBCON (immunomagnetic bead concentrator), a vibrating table used to concentrate the beads in a pellet in the flask. The beads were then collected using the IMBPIP (immunomagnetic bead pipette), a magnetic pipette and re-suspended in a microfuge tube containing 140 µL of PBS.

2.6.2.3 Pathatrix™

The inoculated food samples were placed into a Stomacher™ bag containing 225 mL of citrate buffer. Aliquots (12.5 µL) of each pre-coated A, B, H(2) and H(3) magnetic beads were added to the bags. The samples were mixed by hand for 1 min and then added to the Pathatrix™ machine (Matrix MicroScience Ltd.). The samples were re-circulated for 30 min at room temperature, during which time the beads were collected on the magnet. Beads were washed with 100 mL PBS, collected and transferred in 140 µL PBS to a microfuge tube.

2.7 RNA Extraction

RNA was extracted from 140 µL samples using the QIAamp Viral RNA kit as per manufacturer's instructions (Qiagen, Hilden, Germany). Briefly, the sample was lysed with denaturing buffer AVL containing carrier RNA. After a 10 min incubation at room temperature, absolute ethanol was added to the sample. The sample was then loaded onto a silica-gel membrane column which binds the RNA. The membrane was washed with wash buffers AW1 and AW2 to remove contaminants. RNA was eluted from the membrane in 60 µL of AVE buffer and stored at -20°C until needed.

2.8 Norovirus Detection

2.8.1 Conventional RT-PCR

RT-PCR reactions were performed on a Mastercycler PCR machine (Eppendorf, Hamburg, Germany) using OneStep RT-PCR kit reagents (Qiagen). For norovirus detection, each 25 μL reaction contained 5 μL of RNA, 1X Qiagen OneStep RT-PCR buffer, 400 μM dNTP mix, 400 nM of genogroup specific primers (Table 7), 1μL Qiagen OneStep RT-PCR Enzyme Mix and 30 units of RNase Inhibitor. The cycling condition for the reactions were 42°C for 60 min, 94°C for 15 min and 40 cycles of 94°C for 60 sec, 50°C for 90 sec and 72°C for 60 sec, followed by a final extension at 72°C for 10 min. PCR products were run on a 2 % Agarose gel containing 0.5 X SYBR® Safe (Invitrogen) at 135 V for 60 min.

	Primer/Probe	Sequence (5' to 3')	Reference
Conventional RT-PCR			
GI	G1SKF	CTG CCC GAA TTY GTA AAT GA	(78)
	G1SKR	CCA ACC CAR CCA TTR TAC A	(78)
GII	G2SKF	CNT GGG AGG GCG ATC GCA A	(78)
	G2SKR	CCR CCN GCA TRH CCR TTR TAC AT	(78)
Realtime RT-PCR			
GI	COG1F	CGY TGG ATG CGN TTY CAT GA	(68)
	COG1R	CTT AGA CGC CAT CAT CAT TYA C	(68)
	RING 1A	FAM-AGA TYG CGA TCY CCT GTC CA-BHQ	(68)
	RING 1B	FAM-AGA TCG CGG TCT CCT GTC CA-BHQ	(68)
	RING 1C	FAM-AGA TYG CGI TCI CCT GTC CA-BHQ	(J. Vinjé)[1]
GII	COG2F	CAR GAR BCN ATG TTY AGR TGG ATG AG	(68)
	JJV2F	CAA GAG TCA ATG TTT AGG TGG ATG AG	(67)
	COG2R	TCG ACG CCA TCT TCA TTC ACA	(68)
	N2	FAM-AGA TTG CGA TCG CCC TC-MGBNFQ	(R.M. Ratcliff)[1]
FCV	SH-FCV3-Q-A	GAC ACC TCC GAC GAG TTA TGC	(100)
	SH-FCV3-P	CCG GGT GGG ACT GAG TGG	(100)
	SH-FCV3-Q-1	CY5-CGC CTT ACG GAT ATG AGC AGC CAC ATT AAC-BHQ	(100)

[1]Personal communication.

Table 7. Primers and probes used for the detection of norovirus and FCV

2.8.2 Realtime RT-PCR

2.8.2.1 Genogroup I Norovirus Assay

Realtime RT-PCR reactions were performed on an Mx3005 QPCR system (Stratagene, La Jolla, CA) using Brilliant II QRT-PCR reagents (Stratagene). For GI norovirus detection, each 25 μL reaction contained 2 μL of RNA, 1X CORE RT-PCR buffer, 6 mM $MgCl_2$, 800 μM dNTP mix, 400 nM primers COG1F and COG1R , 60 nM each of the probes RING 1A and RING 1B (Table 7), 1 μL reverse transcriptase, 30 nM ROX reference dye and 1.25 U SureStart® *Taq* DNA polymerase. The conditions were 50°C for 30 min, 95°C for 10 min, followed by 40 cycles of 95°C for 15 sec and 60°C for 60 sec.

Detection of GI norovirus from naturally-contaminated lettuce samples used a slightly modified real time assay with a probe designed to detect a broader range of GI strains. Each 25 μL reaction contained 2 μL of RNA, 1X CORE RT-PCR buffer, 6 mM $MgCl_2$, 800 μM dNTP mix, 400 nM primers COG1F and COG1R, 200 nM probe RING 1C (Table 7), 1 μL reverse transcriptase, 30 nM ROX reference dye and 1.25 U SureStart® *Taq* DNA polymerase. The conditions were 50°C for 30 min, 95°C for 10 min, followed by 40 cycles of 95°C for 15 sec, 55°C for 15 sec and 60°C for 60 sec.

2.8.2.2 Genogroup II Norovirus Assay

Each 25 μL reaction for GII norovirus detection contained 2 μL of RNA, 1X CORE RT-PCR buffer, 5 mM $MgCl_2$, 800 μM dNTP mix, 1 μM primers JJV2F and COG2R, 200 nM probe N2 (Table 7), 1 μL reverse transcriptase, 30 nM ROX reference dye and 1.25 U

SureStart® *Taq* DNA polymerase. Cycling conditions were 50°C for 30 min, 95°C for 10 min, followed by 40 cycles of 95°C for 15 sec, 55°C for 15 sec and 60°C for 60 sec.

2.8.2.3 Feline Calicivirus Assay

Each 25 µL reaction for FCV detection contained 2 µL of RNA, 1X CORE RT-PCR buffer, 5 mM $MgCl_2$, 800 µM dNTP mix, 300 nM primers SH-FCV3-Q-A and SH-FCV3-Q-1, 200 nM probe SH-FCV3-P (Table 7), 1 µL reverse transcriptase, 30 nM ROX reference dye and 1.25 U SureStart® *Taq* DNA polymerase. Cycling conditions were 50°C for 30 min, 95°C for 10 min, followed by 40 cycles of 95°C for 15 sec and 60°C for 60 sec.

2.9 Standard Curve

A standard curve was generated for realtime RT-PCR assays, using RNA transcripts from a plasmid. Amplicons from COG1F and COG1R amplification of a GI.1 strain or from COG2F and COG2R (Table 7) amplification of a GII.4, 2002/Farmington Hills strain were purified using the Wizard® SV Gel and PCR Clean-Up System (Promega, Madison, WI). The purified PCR products were cloned into pCR2.1 using the TOPO TA cloning kit (Invitrogen), according to the manufacturer's instructions. A plasmid containing an FCV insert was obtained from Alain Houde (Agriculture and Agri-Food Canada, St-Hyacinthe, QC). cRNA for GI, GII and FCV standard curves was transcribed as described previously (89), using the MEGAscript T7 high yield transcription kit (Ambion, Austin, TX). Template DNA was removed by incubation with TURBO DNase (Ambion) for 30 min at 37°C. Transcripts were purified with MEGAclear kit (Ambion) and quantified using a NanoDrop™ ND-1000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA). A

standard curve of 10-fold serial dilutions (10^6- 10^{-1} copies) was included with every realtime RT-PCR reaction.

2.10 Lectins

Five µL of streptavidin-coated magnetic beads were dispensed into microfuge tubes. These beads were washed three times with 50 µL of sterile PBS to remove preservatives. For direct binding, sample beads were mixed with multivalent HBGA type A for 30 min at room temperature on a RKDynal rotary mixer. The beads were washed three times with sterile PBS to remove any excess carbohydrates and resuspended in 50 µL of PBS. The conjugated beads were then mixed for 30 min with 5 µg of FITC labelled *Helix aspersa* lectin (HAA) (EY Laboratories Inc., San Mateo, CA). The beads were washed 3 times with 50 µL of PBS to remove any unbound lectins. Indirect binding samples were prepared similarly with HBGA type A mixed with HAA lectin for 30 min, followed by the addition of 5 µg of prewashed beads mixed for 30 min. The conjugated beads from both indirect and direct samples were then resuspended in 100 µL of ultrapure water (Invitrogen) and heated to 70°C for 20 sec to break the biotin-streptavidin linkage (57). The resulting supernatant containing the carbohydrate and fluorescently labelled lectin was then separated from the beads using a magnet. The supernatant was transferred to individual wells of an optically clear QPCR plate (Stratagene). This step was repeated, and the beads were resuspended in ultrapure water and heated to release any remaining lectins. The fluorescence was measured at 516 nm using the quantitative plate reading function on an Mx3005 QPCR system (Stratagene).

2.11 Absorption/Elution Method

Absorption/elution method was adapted from the Health Canada Compendium method for concentrating enteric viruses from water samples (13, 127). Lettuce samples (25 g) were placed in Stomacher™ bags, and diluted 1:10 (w/v) in PBS. The samples were spiked with 50 µL of 6×10^6 PFU/mL FCV as a positive control. The samples were filtered through a positively charged CUNO membrane (Kinecor, Ottawa, ON). The filter was placed in a sterile jar containing 10 mL of tryptose phosphate broth with 6% glycine (pH 9.0) and mixed for 30 min on an orbital shaker to elude any viruses from the filter. The broth was transferred into a 15 mL centrifuge tube, where the pH was adjusted to 7.0 - 7.4 with 1N HCl. The solution was mixed thoroughly and two aliquots of 3.5 mL were added to Amicon Ultra Centrifugal filter units with a 100 kDa membrane (Millipore). The filters were centrifuged at 1610 × g for 20 min. The filter membranes were then washed with 200 µL of PBS, pipeted up and down to elute any viruses. The wash solution from Amicon filter membranes was pooled and 140 µL was removed for RNA extraction.

2.12 Viral Typing

Lettuce samples that tested positive for norovirus by realtime RT-PCR were confirmed by conventional RT-PCR using genotype specific primers (Table 7), as described previously. Any amplicon of the appropriate size that was detected by RT-PCR, was purified and sequenced. Samples with a single amplicon of the correct size were purified using a Microcon YM-50 Centrifugal Filter Unit (Millipore) column. Briefly, 100 µL of H_2O and the remaining PCR product was added to the micron column and centrifuged at 13 000 rpm for 8 min. The column was placed in a new microfuge tube, 30 µL of H_2O was added to the column and vortexed for 10 sec to resuspend the DNA. The column was flipped upside

down and centrifuged at 3 000 rpm for 1 min. For samples with multiple bands, the appropriately sized band was cut from the gel using a razor blade. The DNA was extracted from the fragment using the EZ-10 Spin Column DNA Gel Extraction kit (BioBasics Inc. Markham, ON), following the manufacturers' instructions. The gel fragment was placed in binding buffer II and heated at 60°C for 10 min until completely dissolved. The mixture was added to an EZ-10 column, incubated for 2 min at room temperature and centrifuged at 10 000 rpm for 2 min. The column was washed twice by adding 500 µL of wash solution and centrifuging at 10 000 rpm for 1 min. The column was placed in a new microfuge tube, the DNA was eluted by the addition of 30 µL of elution buffer and incubated for 2 min followed by centrifugation at 10 000 rpm for 2 min.

Forward and reverse sequencing reactions were prepared with the purified DNA using a BigDye Terminator (BDT) v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA). Sequencing BDT reactions contained 5 µL of purified DNA, 3.2 pM of genogroup specific forward or reverse primer (Table 7), 0.875 X BDT buffer and 1 µL BDT mix. Cycling conditions were 96°C for 1 min, 25 cycles of 96°C for 10 sec, 50°C for 5 sec and 60°C for 4 min. The BDT reactions were purified using a Montage SEQ_{96} Sequencing Reaction Cleanup Kit (Millipore). The BDT reaction and 10 µL of injection solution were added to an individual well of the SEQ_{96} plate. The mixture was filtered through the plate using a vacuum system. The filter was washed with 20 µL of injection solution and dried with a vacuum. The sequencing product was resuspended in 25 µL of injection solution and transferred to a new 96 well plate containing 10 µL of Hi-Di formamide in each well. The sequencing reaction was run on an ABI 3130 Genetic Analyzer sequencer (Applied Biosystems).

3 Results

3.1 Preliminary Binding Experiments

Preliminary experiments were done to assess the ability of HBGA carbohydrates to capture norovirus. Univalent biotinylated type A, type B and a mix of type A and B carbohydrates were used to capture norovirus GII.4 2006b/Minerva. Norovirus strain GII.4 2006b/Minerva was chosen for all preliminary experiments, since it was the most common strain circulating worldwide and in Canada during the course of this study (126). Two capture methods were compared, *i.e.*, direct and indirect. For direct capture, carbohydrate-coated magnetic beads were prepared and added to a viral sample. In contrast, for indirect capture, the carbohydrates were first mixed with the viral sample and magnetic beads added to the sample later. Indirect capture allows additional time and freedom for the carbohydrates to interact with the virus. To determine if the beads captured the virus, RNA was extracted from the beads mixture and tested by RT-PCR using primers specific for norovirus. Both direct and indirect capture methods using HBGA type A and B individually and together were successful at capturing norovirus (Table 8).

	HBGA type		
Binding method	A	B	A & B
Direct	+	+	+
Indirect	+	+	+

+ Virus detected by conventional RT-PCR.

Table 8. Preliminary binding experiments using magnetic beads to capture norovirus GII.4 2006b/Minerva

Fifty μg of T1 Dynabeads® and 10 μg of HBGAs were used to capture norovirus from 100 μL of PBS. Test to detect norovirus were done in triplicate by a GII specific conventional RT-PCR assay and scored as positive or negative.

3.2 Optimizing Conditions for Norovirus Capture

3.2.1 Beads

The suitability of four different types of streptavidin coated Dynabeads®, M-280, M-270, MyOne™ Streptavidin C1 and T1 (Invitrogen), for the detection of norovirus, was compared. The beads differed both in size, *i.e.*, 1 μm (MyOne™ Streptavidin C1 and T1) and 2.8 μm (M-280 and M-270), as well as surface properties (hydrophobic and hydrophilic). The beads were used in conjunction with 5 μg of both univalent Blood type A and B carbohydrates via direct or indirect capture methods. The ability of the beads to capture norovirus was tested using a serial dilution series of norovirus GII.4 2006b/Minerva. The presence of norovirus was detected by realtime RT-PCR using specific primers for GII norovirus. Samples were run in triplicate with samples crossing the critical threshold considered positive. All bead types were successful at detecting the higher concentrations of norovirus (Table 9). The M-270 beads were chosen for future work, since they had the most consistent detection of norovirus.

	Bead type							
	M-280		M-270		C1		T1	
Virus dilution	Direct	Indirect	Direct	Indirect	Direct	Indirect	Direct	Indirect
Virus 10^{-1}	2/3[a]	1/3	3/3	3/3	3/3	1/3	1/3	1/3
Virus 10^{-2}	2/3	1/3	3/3	3/3	0/3	0/3	0/3	2/3
Virus 10^{-3}	0/3	1/3	1/3	3/3	0/3	1/3	1/3	0/3
Virus 10^{-4}	1/3	0/3	0/3	0/3	0/3	1/3	1/3	0/3
Virus 10^{-5}	0/3	0/3	0/3	0/3	0/3	1/3	0/3	0/3
No virus	0/3		0/3		0/3		0/3	

[a]Number of triplicates positive by realtime RT-PCR.

Table 9. Detection of 10-fold serial dilutions of norovirus GII.4 2006b/Minerva, using four different types of magnetic beads

Fifty μg of beads and 5 μg of both blood type A and B carbohydrates were used to capture norovirus from 100 μL of PBS. Tests to detect norovirus were done in triplicate by a GII specific realtime RT-PCR assay.

3.2.2 Histo-Blood Group Antigens

The ability of norovirus GII.4 2006b/Minerva to bind different HBGAs was assessed. Various synthetic HBGAs were tested to reflect the natural diversity of HBGA. Both univalent (having a 1:1 ratio of HBGA moiety and biotin) and multivalent (having a 4:1 ratio of HBGA moieties and biotin) carbohydrates were evaluated (Table 5). M-270 beads were used in conjunction with 5 μg of biotinylated HBGA carbohydrates and the direct or indirect capture methods. Negative controls containing no virus or no carbohydrate were included for each HBGA type. Samples with no carbohydrate showed the amount of nonspecific binding of the virus to the beads. Blocking experiments to eliminate nonspecific interactions between the virus and the beads were considered unnecessary as nonspecific interactions also serve to increase the capture of the virus. Samples were tested in triplicate by real-time RT-PCR, and scored positive if they crossed the critical threshold (Ct). The average Ct value is reported for each sample and can be used to approximate the interaction between the carbohydrate and norovirus capsid (Table 10 and Table 11). A standard curve for the realtime assay was developed after these experiments. In general, multivalent carbohydrates were found to bind more strongly to norovirus than univalent carbohydrates. Based on these findings, multivalent carbohydrates were used for all further experiments.

CHO	Negative controls				Direct binding		Indirect binding		Positive control	
	No virus		No CHO		CHO + beads + virus		CHO + virus + beads		Virus only	
	No. pos.	Av. Ct	No. pos.	Av. Ct	No. pos.	Av. Ct	No. pos	Av. Ct	No. pos	Av. Ct
A	0/3[1]	-	1/3	38.67	3/3	36.47	3/3	34.98	3/3	26.31
B	0/3	-	3/3	32.17	3/3	32.61	3/3	32.12	3/3	26.97
H	0/3	-	3/3	33.24	3/3	33.70	3/3	33.20	3/3	28.12
Le^a	1/3	35.33	1/3	36.34	3/3	35.40	1/3	36.77	3/3	30.16
Le^b	0/3	-	1/3	36.44	3/3	35.37	3/3	34.46	3/3	29.66
Le^x	0/3	-	3/3	34.49	3/3	34.16	3/3	32.97	3/3	28.17
Le^y	0/3	-	3/3	36.17	3/3	32.52	3/3	32.94	3/3	27.44

[1]Number of triplicates positive by realtime RT-PCR.

Table 10. Capture of norovirus GII.4 2006b/Minerva using univalent HBGAs

Test to detect norovirus were done in triplicate by a GII specific realtime RT-PCR assay. For positive samples, the average Ct value was reported.

CHO	Negative controls				Direct binding		Indirect binding		Positive control	
	No virus		No CHO		CHO + beads + virus		CHO + virus + beads		Virus only	
	No. pos.	Av. Ct	No. pos.	Av. Ct	No. pos.	Av. Ct	No. pos.	Av. Ct	No. pos.	Av. Ct
A	0/3[1]	-	3/3	34.37	3/3	31.54	3/3	31.10	3/3	28.56
B	0/3	-	3/3	32.56	3/3	32.03	3/3	30.45	3/3	29.55
H (type 2)	0/3	-	2/3	37.53	2/3	37.65	2/3	35.75	3/3	31.38
H (type 3)	0/3	-	1/3	36.24	3/3	34.78	3/3	32.09	3/3	27.73
Le^a	0/3	-	1/3	35.59	2/3	36.03	3/3	33.67	3/3	28.11
Le^b	1/3	38.62	3/3	36.80	3/3	38.06	3/3	36.8	3/3	30.21
Le^x	1/3	39.40	2/3	37.18	3/3	37.79	3/3	35.82	3/3	30.14
Le^y	0/3	-	1/3	36.36	3/3	31.68	3/3	30.89	3/3	27.80

[1]Number of triplicates positive by realtime RT-PCR.

Table 11. Capture of norovirus GII.4 2006b/Minerva using multivalent HBGAs

Tests to detect norovirus were done in triplicate by a GII specific realtime RT-PCR assay. For positive samples, the average Ct value was reported.

3.2.3 Amount of Carbohydrates

To ensure that the beads were completely saturated with carbohydrates, experiments were done with fluorescently labelled lectins. Lectins are protein molecules which recognize and bind to specific carbohydrate moieties. To simulate direct and indirect binding, the lectins were mixed with pre-coated carbohydrate conjugated beads, as well as carbohydrates, prior to the addition of the beads. The level of fluorescence was indicative of the amount of carbohydrates conjugated to the beads. The results indicate that 1 μg of biotinylated carbohydrate will saturate 5 μg of M-270 Dynabeads® when using direct binding (Figure 6). The results were similar for indirect binding. The higher and more variable fluorescence values are likely due to the clustering of multiple lectins to the carbohydrates, since they had more freedom to interact.

An identical experiment was completed for streptavidin coated beads purchased from Matrix MicroScience. These beads were specifically designed for use in the Pathatrix™, after it was discovered that the Dynabeads® used previously were not compatible with the Pathatrix™ system. The results for the matrix beads also indicated that 1 μg of biotinylated carbohydrate will saturate 5 μL of beads, addition of more carbohydrates did not considerably increase the fluorescence (Figure 7).

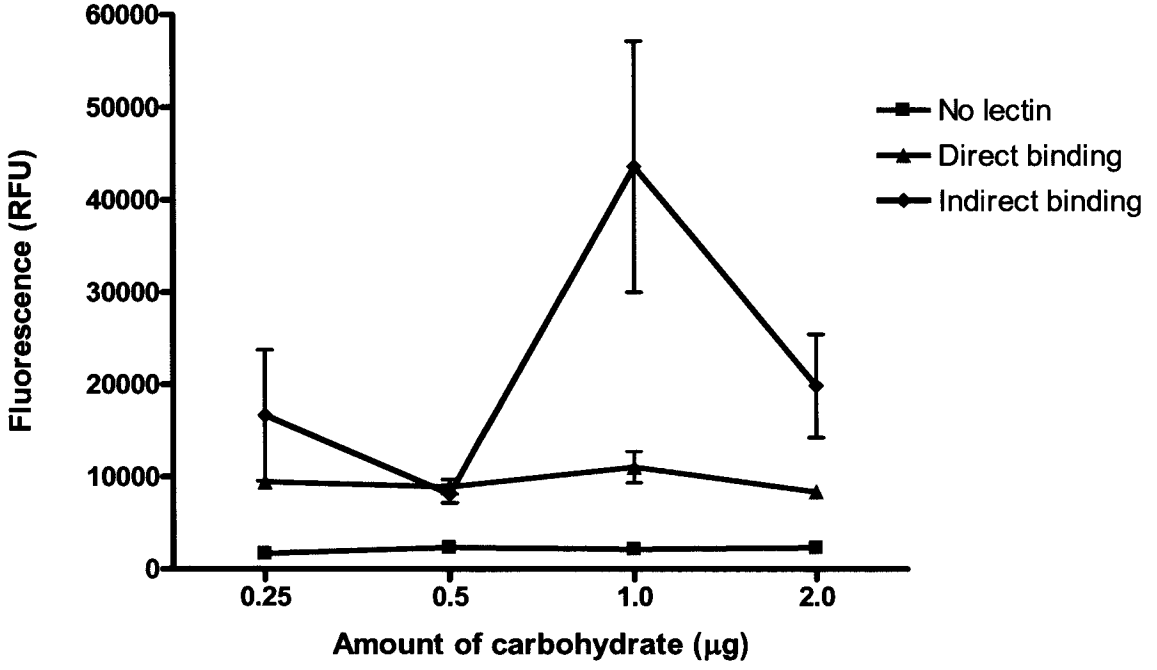

60000
50000
40000
30000
20000
10000
0
Fluorescence (RFU)
0.25
0.5
1.0
2.0
Amount of carbohydrate (μg)
No lectin
Direct binding
Indirect binding

Figure 6. Binding of biotinylated carbohydrates to Dynabeads®

Graph shows the fluorescence measured in relative fluorescence wnits (RFU) detected using different amounts of carbohydrates (measured in micrograms) under different binding conditions. The red line with diamonds shows indirect binding, blue line with triangles shows direct binding and the green line with squares shows binding with no lectins.

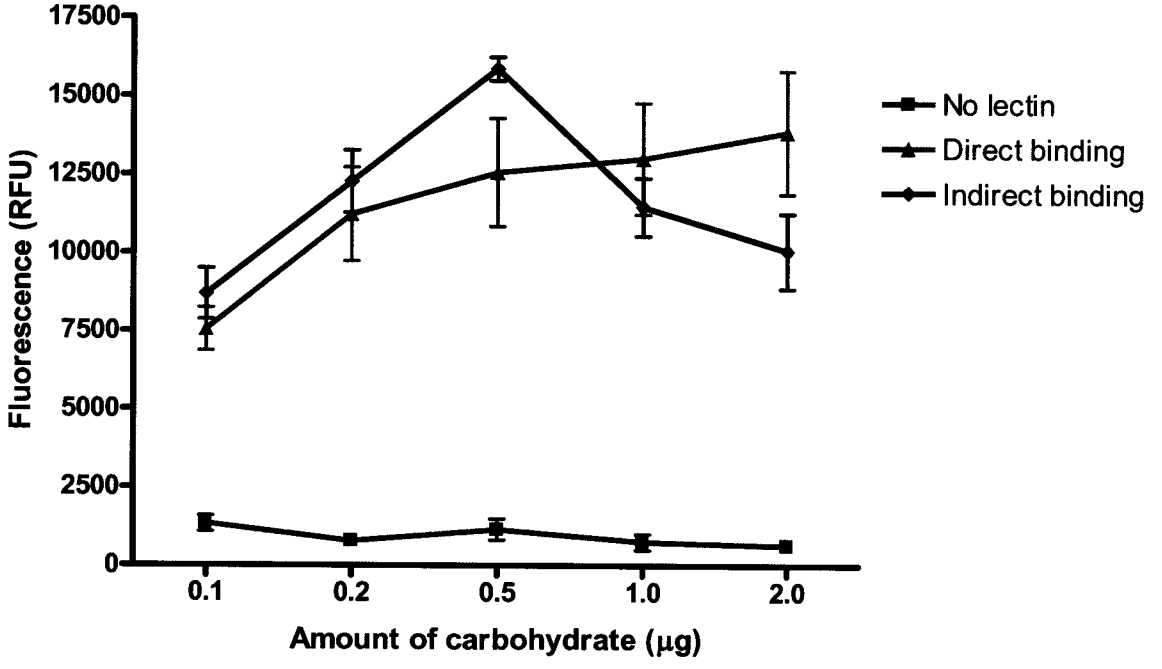

17500
15000
12500
10000
7500
5000
2500
0
Fluorescence (RFU)
0.1
0.2
0.5
1.0
2.0
Amount of carbohydrate (μg)
No lectin
Direct binding
Indirect binding

Figure 7. Binding of biotinylated carbohydrates to Matrix beads

Graph shows the fluorescence measured in relative fluorescence wnits (RFU) detected using different amounts of carbohydrates (measured in micrograms) under different binding conditions. The red line with diamonds shows indirect binding, blue line with triangles shows direct binding and the green line with squares shows binding with no lectins.

3.3 Optimizing Large Volume Capture

3.3.1 Selection of Buffer

After sufficiently optimizing the capture of norovirus from small volumes, the capture of norovirus from larger volumes (250 mL) was tested. Two different systems were used for the concentration of magnetic beads from 250 mL of buffer, the Pathatrix™ and *i*CropTheBug. A variety of different buffers covering a range of pH values were tested (Table 12). Direct binding, with pre-coated beads added to the buffer for capture of norovirus was used to test all the buffers. Citrate buffer (pH 4.0) with or without peptone was the only buffer successfully used to capture norovirus in the *i*CropTheBug system. Therefore, citrate buffer (pH 4.0) was selected for all further experiments. The Pathatrix™ system was unsuccessful at capturing norovirus using all buffers (Table 12). This was most likely due to the incompatibility of the Dynabeads® with the Pathatrix™ system.

Buffer	pH	Pathatrix™	Virus input[a]	*i*CropTheBug	Virus input
Citrate	3.0	-	1780	-	1713
Citrate with 1% peptone	3.0	-	270	-	276
Citrate	4.0	-	1780	+	1713
Citrate with 1% peptone	4.0	-	270	+	276
Citrate	5.0	-	1780	-	1713
Citrate with 1% peptone	5.0	-	270	-	276
Citrate	6.0	-	1780	-	1713
Citrate with 1% peptone	6.0	-	270	-	276
Buffer phosphate water	7.2	-	4240	-	3060
PBS	7.2	-	863	-	1090
Glycine buffer	9.0	-	1480	Not done	Not done

[a]The amount of norovirus added to 250 mL of buffer, as quantified by realtime RT-PCR.
- Norovirus not detected by realtime RT-PCR.
+ Norovirus detected by realtime RT-PCR.

Table 12. Capture of norovirus GII.4 2006b/Minerva from 250 mL of various buffers

Tests to detect norovirus were done in triplicate by a GII specific realtime RT-PCR assay and scored as positive or negative.

3.3.2 Direct Versus Indirect Capture

The efficiency of direct and indirect capture of norovirus using HBGA conjugated magnetic beads in large volumes was investigated. M-270 Dynabeads® were used in conjugation with multivalent blood type A biotinylated HBGA to capture norovirus (10^4 to 10^1 genome equivalents) in 250 mL of citrate buffer. The beads were concentrated using two different automated systems,*i.e.*, *i*CropTheBug and Pathatrix™. The Pathatrix™ system was unable to detect any norovirus, most likely due to the incompatibility of Dynabeads® with the system, as mentioned previously (Table 13). The *i*CropTheBug was able to detect norovirus using both direct and indirect binding. Indirect binding originally improved capture in small volumes, possibly because it allowed more freedom and time for the interaction between the HBGAs and norovirus (Table 10 and Table 11). However in large volumes, there was no clear advantage to indirect binding (Table 13). Direct binding was selected for future work since it enabled the beads to be prepared in advance for ease of use and quality control.

		Virus Input (copies/250 mL)			
		10^4	10^3	10^2	10^1
Pathatrix™	Direct	0/2[a]	0/3	0/3	0/1
	Indirect	0/1	0/3	0/2	Not done
*i*CropTheBug	Direct	2/2	1/3	0/3	0/3
	Indirect	1/1	0/3	1/2	Not done

[a]Number of replicates positive for norovirus by realtime RT-PCR out of the total number of replicates.

Table 13. Capture of norovirus GII.4 2006b/Minerva using direct and indirect binding

Tests to detect norovirus were done in triplicate by a GII specific realtime RT-PCR assay.

3.4 FCV as a Positive Control

FCV is commonly used as a positive control in food virology detection methods, due to its similarity to norovirus (100). The applicability of FCV as a positive control for the carbohydrate-conjugated beads method was evaluated. A mixture of 50 µL of A, B, H(2) and H(3) HBGA-conjugated magnetic beads did not bind any FCV at input levels of 3×10^5 PFU, when tested by a realtime RT-PCR specific for FCV. This indicated that the addition of FCV would not interfere with the detection of norovirus. To capture FCV, sialic acid conjugated magnetic beads were created as was done previously for HBGAs (see 2.6.2.1 Carbohydrate-Conjugated Magnetic Beads). Sialic acid has been shown to bind to FCV and MNV but not human noroviruses (62, 133, 139). The sialic acid conjugated beads were successful at detecting FCV from both small and large volumes of 100 µL and 250 mL, respectively (Table 14). When FCV was added as a positive control to 25 g samples of lettuce, 10 µL of sialic acid conjugated beads were able detect FCV at all input levels. The percent recovery was calculated based on the quantity of virus recovered and the virus input quantity, determined by realtime RT-PCR. At all input levels, the percent recovery failed to reach the minimum threshold of 1% (32). Doubling the amount of sialic acid beads added failed to increase the percent recovery (Table 15).

Cationic beads which have been successfully used to capture FCV in other methods were also tested. However, they failed to capture FCV at all input levels when used in this system (Table 15).

	Volume	
Virus input (PFU)	Small volume (100 μL)	Large volume (250 mL)
3×10^5	+	+
3×10^4	+	+
3×10^3	+	+
3×10^2	Not done	+
0	-	-

+ FCV detected by realtime RT-PCR.
- FCV not detected by realtime RT-PCR.

Table 14. Capture of FCV with sialic acid conjugated beads

50 μL of sialic acid conjugated magnetic beads were used to detect various inputs of FCV from small volumes (100 μL) of PBS and large volumes (250mL) of citrate buffer using the *i*CropTheBug System. Test to detect FCV were done in triplicate by realtime RT-PCR assay and score as positive or negative.

Virus titre (PFU)	10 μL Sialic acid beads		20 μL sialic acid beads		20 μL cationic beads	
	No. pos.	% recovery	No. pos.	% recovery	No. Pos.	% recovery
3×10^5	3/3[a]	0.135 %[b]	2/3	0.000 %	0/3	-
3×10^4	3/3	0.387 %	3/3	0.010 %	0/3	-
3×10^3	3/3	0.248 %	3/3	0.011 %	0/3	-
0	0/3	-	0/3	-	0/3	-

[a]Number of replicates positive for FCV by realtime RT-PCR out of a total of 3 replicates.
[b]Percent recovery of FCV based on FCV recovery by realtime RT-PCR and virus input.

Table 15. Detection of FCV used as a positive control from 25 g samples of lettuce using sialic acid or cationic beads

Tests to detect FCV were done in triplicate by a FCV specific realtime RT-PCR assay.

3.5 Method Validation

3.5.1 Norovirus Detection in Buffer

HBGA Type A-conjugated beads were used to detect GII.4, 2006b/Minerva norovirus in 250 mL of citrate buffer (Table 16). The Pathatrix™ and the *i*CropTheBug system were both able to detect a minimum of 10^2 copies / 250mL. Since not all norovirus strains bind to HBGA Type A, additional HBGAs (Types B, H(2) and H(3)) were conjugated to beads. A mixture of A, B, H(2) and H(3)-conjugated beads were tested in experiments to detect a GII.4, 2006b/Minerva norovirus in 250 mL of citrate buffer (Table 16). The minimum detection limit was 10^2 copies /250 mL for both systems. These results suggest that the mixture of A, B, H(2) and H(3)-conjugated beads did not alter the limit of detection for the assay.

System	HBGA	Input virus (copies / 250 mL) No. positive replicates (N=3)			
		10^4	10^3	10^2	10^1
*i*CropTheBug	A	2/3[a]	2/3	1/3	Not done
	A, B, H(2), H(3)	3/3	2/3	1/3	Not done
Pathatrix™	A	2/3	2/3	2/3	Not done
	A, B, H(2), H(3)	Not done	3/3	1/3	0/3

[a]Number of replicates positive for norovirus out of a total of three replicates.

Table 16. Detection of norovirus GII.4, 2006b/Minerva in buffer using A-conjugated magnetic beads and A, B, H(2) and H(3)-conjugated magnetic beads with two automated extraction systems

For each dataset, the three viral additions were undiluted, 1/10 and 1/100. The input titre was calculated for each experiment and varied due to differences in the stock sample. Tests to detect norovirus were done in triplicate by a GII specific realtime RT-PCR assay.

3.5.2 Norovirus Detection from Food

Food products (25 g) were inoculated with 10-fold serial dilutions of a GII.4, 2006b/Minerva norovirus. The virus was concentrated using A, B, H(2) and H(3)-conjugated beads and either the *i*CropTheBug or Pathatrix™ (Table 17). The minimum level of detection increased for most food samples compared with experiments in buffer. Using the *i*CropTheBug, the detection limit for norovirus on green onions remained the same as in buffer (10^2 copies/250 mL). Virus was detected from lettuce and deli ham at a minimum input level of 10^3 copies/250 mL and on strawberries at 10^4 copies/250 mL. The Pathatrix™ had a lower minimum limit of detection (10^1 copies/250 mL) for lettuce, green onions and strawberries when compared with the *i*CropTheBug, however detection using the Pathatrix™ was less reliable. For lettuce, green onions and strawberries samples were sporadically positive, across a large range of input titres. Consistent detection of positive samples was not achieved even at the highest input levels (Table 17). Virus was not detected from any deli ham sample ($10^1 - 10^3$ copies/250 mL) using the Pathatrix™.

System	Matrix	Input virus (copies/250 mL) No. positive replicates (N=3)			
		10^4	10^3	10^2	10^1
*i*CropTheBug	Buffer	3/3[1]	2/3	1/3	Not done
	Lettuce	3/3	1/3	0/3	Not done
	Green onions	Not done	2/3	1/3	0/3
	Strawberries	1/3	0/3	0/3	Not done
	Deli-ham	2/3	1/3	0/3	Not done
Pathatrix™	Buffer	Not done	3/3	1/3	0/3
	Lettuce	Not done	1/3	1/3	1/3
	Green onions	Not done	1/3	1/3	1/3
	Strawberries	Not done	0/3	0/3	1/3
	Deli-ham	Not done	0/3	0/3	0/3

[1]Number of replicates positive for norovirus out of a total of three replicates

Table 17. Detection of norovirus GII.4, 2006b/Minerva in buffer and food products using A, B, H(2) and H(3)-conjugated magnetic beads with two automated extraction systems

For each dataset, the three viral additions were undiluted, 1/10 and 1/100. The input titre was calculated for each experiment and varied due to differences in the stock sample. Tests to detect norovirus were done in triplicate by a GII specific realtime RT-PCR assay

3.5.3 Detection of Multiple Norovirus Strains

The carbohydrate-conjugated bead method was also tested with additional norovirus strains. The combination of A, B, H(2) and H(3) conjugated beads was able to detect all 9 strains tested (Table 18). The *i*CropTheBug detected the strains with a minimum level of detection in the range of 10^0 to 10^2 copies/250mL. Detection with the Pathatrix™ was more variable, with a minimum level of detection in the range of 10^0 to 10^3 copies/250 mL. One strain, GI.13, was not detected in the Pathatrix™ (Table 18).

System	Strain	Input virus (copies/250 ml) No. positive replicates (N=3)				
		10^4	10^3	10^2	10^1	10^0
*i*CropTheBug	GI.1	Not done	3/3[a]	3/3	1/3	0/3
	GI.3b	Not done	Not done	1/3	1/3	1/3
	GI.13	3/3	3/3	Not done	1/3	0/3
	GII.2	3/3	1/3	2/3	0/3	Not done
	GII.3	3/3	3/3	3/3	1/3	0/3
	GII.4 2002	3/3	3/3	1/3	1/3	Not done
	GII.4 2004	3/3	2/3	Not done	2/3	0/3
	GII.4 2006a	3/3	3/3	2/3	Not done	0/3
	GII.4 2006b	3/3	2/3	1/3	Not done	Not done
Pathatrix™	GI.1	3/3	3/3	3/3	0/3	Not done
	GI.3b	3/3	3/3	0/3	Not done	Not done
	GI.13	Not done	0/3	0/3	0/3	Not done
	GII.2	Not done	3/3	2/3	1/3	0/3
	GII.3	Not done	Not done	3/3	3/3	1/3
	GII.4 2002	3/3	3/3	1/3	1/3	Not done
	GII.4 2004	Not done	Not done	1/3	0/3	0/3
	GII.4 2006a	3/3	1/3	0/3	0/3	Not done
	GII.4 2006b	Not done	3/3	1/3	0/3	Not done

[a]Number of replicates positive for norovirus out of a total of three replicates.

Table 18. Detection of multiple norovirus strains in buffer using A, B, H(2) and H(3)-conjugated magnetic beads with two automated extraction systems

The input titre was calculated for each experiment and varied due to differences in the stock sample Tests to detect norovirus were done in triplicate by a GI or GII specific realtime RT-PCR assay.

Strain	Source	Location	Date
GI.1	Oyster outbreak	British Columbia	2004
GI.3b	CDC stock[1]	United States	2007
GI.13	Cruise ship	Vancouver, BC	2004
GII.2	Cruise ship	Vancouver, BC	2004
GII.3	Catered event	Maple Ridge, BC	2004
GII.4 2002	Outbreak	Vancouver, BC	2004
GII.4 2004	CDC stock	United States	2007
GII.4 2006a	Family outbreak	Gatineau, QC	2007
GII.4 2006b	Catered event	Winnipeg, MB	2007

[1]Center for disease control (CDC)

3.5.4 Detection of Naturally-Contaminated Samples

Testing of lettuce samples was done to evaluate the ability of the carbohydrate-conjugated bead method to detect natural contamination. Samples were tested with both the carbohydrate-conjugated bead method developed in this thesis, and a standard absorption/elution method from the Health Canada Compendium using positively charged filters to concentrate any virus present. RNA was extracted from the concentrated viruses and realtime RT-PCR methods were used to test for GI and GII norovirus (see 2.7 RNA Extraction and 2.8.2 Realtime RT-PCR). Four samples were positive for norovirus out of a total of 110 samples (Table 19). Sample number 5 was positive for GI.2 by the absorption/elution method and a GII.4 strain by the carbohydrate conjugated bead method. Sample number 6 was positive for GI.6 and GII.4 2006b/Minerva by the absorption/elution method. Sample number 10 was positive for GII.4 2006b/Minerva by the carbohydrate-conjugated bead method. Sample number 42 was positive for GI.2 by the absorption/elution method. Finally, sample number 90 was positive for GI.4 by the absorption/elution method.

Table 19. Detection of genogroup I and II norovirus from naturally-contaminated lettuce using both carbohydrate-conjugated magnetic beads and absorption/elution method

	Carbohydrate-conjugated beads		Absorption/elution	
Genotype	No. pos.	Strain	No. pos.	Strains
GI	0	-	4	GI.2, GI.4 & GI.6
GII	2	GII.4 & GII.4 2006b	1	GII.4 2006b

A total of 110 samples were analyzed by both methods.

4 Discussion

4.1 Method Development

The norovirus detection method developed in doing this thesis work used HBGA conjugated magnetic beads to capture norovirus particles present in the sample. This proved to be an efficient system for concentrating virus particles and removing inhibitory molecules, which are important factors when using downstream applications such as realtime RT-PCR. This method represents a significant improvement over previously published methods, *i.e.*, it is rapid, easily transferable to new labs and is sensitive enough to detect low levels of virus as well as multiple strains. In order to optimize the detection limits of the assay, several variables in the method were carefully considered.

Streptavidin coated Dynabeads® were selected for this assay. Dynabeads® are commercially available superparamagnetic beads with a polymer shell. The beads are highly uniform in size and shape. The streptavidin coating of these beads forms a strong non-covalent bond to biotin molecules on synthetic HBGA carbohydrates. The commercial availability of both these items makes this method both highly reproducible and easily transferable to other labs. Biotinylated HBGAs are available in univalent and multivalent forms. Both successfully captured norovirus, however, as measured by semi-quantitative realtime RT-PCR assay the multivalent carbohydrates recovered more virus. The longer linker chain between the biotin molecules and carbohydrate moiety may have allowed for more efficient interactions between the viral particles and the coated beads by preventing steric interference. In addition, the multivalent chains had a 4:1 ratio of carbohydrate

moieties and biotin molecule, whereas univalent chains had a 1:1 ratio. This increased the number of carbohydrate moieties per bead available for binding to norovirus particles.

The majority of enteric virus detection methods being used today involve the use of buffer to elude the viruses from foods. These buffers usually have a neutral or alkaline pH (7.0 to 9.5). However, in the carbohydrate-conjugated beads method, acidic citrate buffer with a pH of 4.0 resulted in the best detection. Our findings were confirmed by a related study using porcine gastric mucin (containing HBGAs) to capture norovirus, which showed that there was a considerable increase in non-specific binding at low pH. This non-specific binding acts in addition to specific binding, resulting in maximum capture of norovirus at pH 3.0-4.2 (143).

Direct binding was selected for the final protocol because it had the advantage of allowing beads to be prepared ahead of time for quality control and consistency. Indirect binding allows more time and freedom for weak interactions between the carbohydrate and norovirus to occur. Indirect binding showed a slight advantage over direct binding in small volumes, but there was no advantage when using 250 mL. This may have been due to the decreased likelihood of carbohydrate–bead interactions in the larger volume.

4.1.1 Internal Control

An internal or sample process control is added to the samples prior to processing to ensure the method is completed properly. If the control is not detected or detected at a lower level than expected, this suggests problems with the method or the presence of inhibitors in the sample. Thus, the addition of an internal control to a method can prevent the reporting of false-negatives. Health Canada has proposed the use of FCV as an internal control for

enteric virus analysis. FCV was selected due to its similarity to enteric viruses, limited distribution in the environment and established methods propagation and plaque assay (100).

Since the carbohydrate-conjugated bead method was developed for the specific concentration of norovirus, additional beads were required to capture FCV. Sialic acid conjugated beads, captured FCV, but failed to obtain a 1% recovery. This has been suggested as the minimum acceptable recovery for an internal control (32). This low recovery may have been due to a low affinity between sialic acid and FCV. Sialic acid is not the functional receptor for FCV entry, and the biological significance of this interaction is unknown (92). It is also possible that the low recovery may have been due to the mechanics of the method, requiring intricate binding to occur between a relatively small number of virus particles and carbohydrates in a large volume of liquid. The percentage recovery of norovirus in the *i*CropTheBug system ranged from 0.02 - 99% with a median of 1.08%. One way to overcome this difficulty may be to quantify the detection limit for FCV and then use this information to determine the appropriate spike levels for the validation of method performance (100).

In order to understand the reasons for the variable recoveries of FCV and norovirus strains from buffer, additional experiments could be done to measure the affinity of norovirus and FCV for their carbohydrate ligand. For example, this could be accomplished using surface plasmon resonance technology to measure the dissociation constant of purified viral capsids with bound ligand (23). The measured affinity would likely be highly dependent on the strain of norovirus and type of HBGA. An understanding of the affinities of various norovirus strains for a range of HBGA molecules could be used to improve the capture of norovirus by the carbohydrate-conjugated beads method.

The use of cationic beads with the carbohydrate-conjugated bead method to capture the FCV internal control was also evaluated. Cationic beads are positively-charged, and have been used successfully to capture both hepatitis A virus and FCV in the Pathatrix™ system (100, 110). However, FCV was not captured using citrate buffer and the *i*CropTheBug system. A visual examination of the pellet which was formed confirmed that the cationic beads were captured by the *i*CropTheBug. The failure to capture FCV could possibly be explained by differences in buffer pH, *i.e.*, these beads successfully captured FCV in a buffer of pH 9.0, while our method used a buffer of pH 4.0. The citrate buffer is below the isoelectric point (pI) of FCV, reported to be 4.9 (149). Therefore, at the pH 4.0 used in this system, FCV would be predicted to have a net positive charge and could have been repelled from the cationic beads.

4.2 Method Validation

4.2.1 Detection from Buffer and Food Products

To capture multiple norovirus strains, a mixture of A, B, H(2) and H(3) HBGA conjugated beads were used. Based on the measured binding patterns, the majority of norovirus strains are known to bind one or more of these antigens (58). Some strains have been identified that only bind Lewis antigens, however, these were not available in our lab. A mixture of all 9 HBGA antigens was not used, due to concerns that too many antigens would interfere with the detection of the more common A/B binding group strains. Specifically, the most prevalent norovirus strain circulating in Canada at the time of this study (GII.4 2006b/Minerva) was shown to bind antigens in the A/B group (17). The number and type of antigens used in the method developed in this study could easily be

adjusted to take into account strain variation over time. The method as developed, detected three strains from genogroup I and six from genogroup II, including four different GII.4 variants. Although the GI.13 strain was not captured in the Pathatrix™ system, it was effectively captured with the *i*CropTheBug, suggesting that an error occurred during processing. Thus, the lack of detection apparently was not due to incompatibility of that particular strain with the HBGAs used to coat the beads.

Lettuce, green onions, strawberries and deli-ham were selected for method validation based on their availability and association with past outbreaks of enteric virus infection. Only one norovirus strain, GII.4 2006b/Minerva, was used to assess detection from food products, due to limited supplies of the other strains. In general, the detection of norovirus from food was less sensitive and consistent than detection of norovirus from buffer. This is likely due to the complex and varied nature of food matrices which may have affected the recovery of the beads. The different chemical and physical nature of the food products tested also likely affected viral recovery. Factors such as pH, salt concentration, electrostatic and hydrophobic forces have all been shown to influence viral attachment to food products (148). As a result, some food products allow for better virus recovery than others. This underscores the importance of having an internal control in detection methods.

Virus inputs were quantified based on realtime RT-PCR Ct values as compared with a standard curve of cRNA copies. It is important to note that virus input may not necessarily represent intact or infectious virus. Damage to the virus capsid may have occurred during freezing of samples for storage. Any changes in the capsid would affect the virus's ability to bind to HBGA molecules, but still protect the genome from degradation.

4.2.2 Pathatrix™ Versus *i*CropTheBug

In order to concentrate virus from food products a magnetic separation system was needed which was capable of handling large volumes of 250 mL. The standard sample size for food microbiology is 25 g, diluted 1:10 (w/v) in buffer for a total amount of 250 mL. Two magnetic separation systems were available and were capable of handling this volume, *i.e.*, the Pathatrix™ and *i*CropTheBug. The Pathatrix™ system is commercially available from Matrix Microscience and has been used successfully to detect bacteria and viruses (104, 110, 152). The major advantage of the Pathatrix™ is that it could run up to five samples simultaneously. However, it requires the purchase of consumables for each reaction and the magnetic beads compatible with the system are a proprietary technology. The *i*CropTheBug was simple to use and compatible with multiple bead types, but each flask was processed individually. The *i*CropTheBug provided more consistent detection of norovirus from food samples. The inconsistency of the Pathatrix™ for the detection of norovirus is thought to be due to difficulties capturing the beads. Since the beads have to travel from the food sample well through tubing to reach the magnet, there are more opportunities for the beads to be lost during the collection run. There was also a considerable cost difference at $8 per sample for the *i*CropTheBug and $30 per sample for the Pathatix™. Based on the results from this research, the *i*CropTheBug was the best system for the detection of norovirus with HBGA-coated beads. The method developed here could also be applied to other magnetic separation systems, as they become available.

4.3 Comparison to Other Methods

During the course of this study, two other methods were published using HBGA-coupled magnetic beads to detect norovirus (19, 144). One paper used porcine gastric mucin, which contains HBGA Types A, H(1) and Lewis B, as a norovirus capture reagent (144). A second protocol was based on synthetic HBGA Type H(1) conjugated to magnetic beads (19). This study was limited in its scope, testing only one norovirus strain and a small sample volume as a proof of principle. The work presented here is the first to demonstrate the use of HBGA-conjugated beads for the extraction of multiple norovirus strains. In addition, the reagents have been tested in automated concentration systems and with a variety of food products.

The sensitivity of the carbohydrate conjugated bead method was compared to a standard absorption/elution method from the Health Canada Compendium. This method was chosen for comparison because of its ability to detect other enteric viruses including rotavirus, which was required for a parallel study on the same samples. The absorption/elution method detects multiple viruses using a nonspecific positively-charged filter that adsorbs enteric virus capsids. This method is time consuming and requires expensive filters which have limited availability. In addition, it can only be used for non viscous liquids or food samples which can easily be washed with buffer.

Comparison of the absorption/elution method and the HBGA method showed that both methods detected noroviruses from naturally-contaminated samples. In total only four samples were positive for norovirus out of a total of 68, *i.e.*, four by absorption/elution and two by HBGA extraction. The low number of positive samples is consistent with previous surveillance studies for enteric viruses (1, 55). Only one lettuce sample was positive for

norovirus by both methods, and different strains were detected by each method. This may be explained by the fact that two different 25 g subsamples were taken to complete both methods. Norovirus is thought to be present at low levels and not evenly distributed in contaminated food samples. Although there was a small sample size, the methods appear to perform similarly.

One other method is available for norovirus detection in the Health Canada Compendium. This method uses a polyethylene glycol step to concentrate the virus, followed by RNA extraction and purification using TRIzol® and poly (dT) magnetic beads before detection with RT-PCR. This method is difficult and time consuming, taking two days to complete. Previous work in the lab showed that this method was susceptible to inhibitors from food and was less sensitive than cationic beads for viral detection (113).

4.4 Conclusions and Future Work

The HBGA-conjugated bead protocols developed here for the extraction and concentration of norovirus from food matrices provide a rapid and effective tool for investigating the suspected contamination of ready-to-eat food products with norovirus. The method proved to be effective for the detection of norovirus from both spiked and naturally contaminated samples. The standardized reagents and automated extraction systems involved are readily available for implementation in food testing laboratories. The entire method, using either the Pathatrix™ system or *i*CropTheBug can easily be completed in one day.

Future work will refine the internal control procedure to make this method suitable for publication in volume 5 (Methods for the Analysis of Parasites, Viruses and Other Foodborne Pathogens) of the Health Canada Compendium of Analytical Methods for the microbiological evaluation of food products. Also, this HBGA-conjugated bead method may be improved by investigating the factors that effect virus attachment to food products and the addition of sample pre-treatment methods such as filtration and centrifugation to limit the interference of food products in virus capture. This method can be used to test any food samples linked to norovirus outbreaks that may occur.

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