Evaluation of norovirus persistence on farm and agriculturally-relevant environments

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Abstract

Human norovirus (NoV) causes gastroenteritis worldwide and has been associated with a number of produce related outbreaks. The design of effective inactivation and prevention procedures requires an understanding of virus survival in environments applicable to the production and processing of fresh produce.

To evaluate the extent of NoV risk from farm to fork, the survival of murine norovirus (MNV), a surrogate for human NoV, was studied on stainless steel disks, soil and in bottled water for 42 days and on lettuce for 15 days in the laboratory. Stability experiments were then conducted on farm during one lettuce planting/harvest cycle, for 4 weeks.

MNV stability was tested at room temperature in the laboratory or under ambient conditions on the farm. A one log reduction in virus titre was achieved after 30 days in water, 4 days on lettuce, 15 days on stainless steel disks, 12 days on loamy and sandy soil. For farm testing, infectious virus was recovered from both soil and lettuce on the day of inoculation. Although infectious virus was not recovered at later time points, the viral genomes were detected for up to four weeks.

The observed long-term persistence of NoV, under both laboratory and field conditions, provides valuable information for developing risk assessments and control procedures to limit the possibility for NoV transmission in the food supply.

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List of Abbreviations

ACP	Amorpous calcium phosphate
AGI	Acute gastroenteritis
BDT	BigDye^{TM} terminator
BSA	Bovine serum albumin
DMEM	Dulbecco's modified eagle's medium
EBSS	Earl's balanced salt solution
FBS	Fetal bovine serum
FCV	Feline calicivirus
GG	Genogroup
GI	Genogroup I
GII	Genogroup II
HAV	Hepatitis A virus
HBGA	Histo-blood group antigens
IEM	Immune electron microscopy
MOI	Multiplicity of infection
MNV	Murine norovirus
NLV	Norwalk like virus
NoV	Norovirus
ORF	Open reading frame
PBS	Phosphate buffered saline
PFU	Plaque forming unit

RDRP	RNA dependent RNA polymerase
RT-PCR	Reverse transcription polymerase chain reaction
ssRNA	Single stranded RNA
SRSV	Small round structured viruses
TPBG	Tryptose phosphate broth glycine
UV	Ultraviolet

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1 Introduction

1.1 Food safety

Food safety has become an increasingly important public health priority due to recent large, publicized outbreaks (78, 93, 124). Outbreaks caused by foodborne contaminants involve people of all age groups and affect not only people's health, but they also have a significant economic impact. Foodborne diseases in the United States of America (USA) are estimated to cost up to $35 billion annually. For example, a NoV outbreak in 2007 cost $650,000 at Johns Hopkins hospital (72).

Foodborne diseases are a public health concern in both developed and developing countries. According to the World Health Organization (WHO), the burden of foodborne diseases in developed countries is estimated at 2.2 million deaths annually which was largely attributed to the consumption of contaminated water and food (90). Scallan *et al.*, (2011), reported that out of 9.4 million cases of foodborne illness estimated every year in the USA, 55,961 lead to hospitalizations and 1,351 lead to death (104), with human NoV being responsible for 58% of illnesses, 35% of hospitalizations and 11% of deaths. According to the Government of Canada, up to 11 million cases of foodborne diseases are estimated every year (115).

The International Commission on Microbiological Specifications for Foods (ICMSF) defines food safety objective as "The maximum frequency and/or concentration of a [microbiological] hazard in a food at the time of consumption that provides the appropriate level of protection (ALOP)". To achieve this goal, all the stages of food production, from

farm to fork, have to be effectively monitored and assessed as contamination can occur anywhere in the chain and be transmitted to an individual and cause illness.

Food has long been considered a potential vehicle for pathogens. Food can be contaminated either accidentally or intentionally. The detection of contaminants can become much more challenging when the contaminant is not one of the agents which is routinely monitored in the food protection and surveillance studies.

Some of the most common foodborne bacterial pathogens are: *Salmonella spp.*, *Campylobacter spp.*, *Escherichia coli* O157, and *Listeria monocytogenes.* The most important foodborne viruses are human NoV and Hepatitis A virus (HAV).

1.2 Foodborne Viruses

Viruses can enter the human population and cause disease in many different ways including through food and water. Foodborne viruses usually infect the cells of the host's gastrointestinal tract, cause diarrhea, and are shed in high numbers via the stool of the infected persons. Known foodborne or waterborne viruses include NoV, HAV, enteric adenovirus, rotavirus, sapovirus, astrovirus, coronavirus and aichivirus (Table 1). Of all the foodborne viruses, human NoV is the leading cause of acute gastroenteritis worldwide (87).

The first report of a viral foodborne outbreak, caused by poliovirus, was documented in 1914, related to the consumption of raw milk. This disease was later prevented through the development of a specific vaccine (102). However, more viral foodborne outbreaks were reported globally and the number of such incidences is rapidly growing (64). Viruses are being introduced into the food processing system more frequently, or are detected in patients more rapidly, which is why their survival and inactivation on/in food products is an interesting and important area of research.

There are some fundamental features exclusive to foodborne viruses that differentiate them from foodborne bacteria. First, viruses are present in low numbers and do not grow on food products since viruses require living cells to replicate. Foodborne bacteria, however, do continue to grow and multiply and may change the texture of the food (71). Secondly, most foodborne viruses are human pathogens, highly infectious and able to spread rapidly through person-to-person contact whereas foodborne bacteria may infect a wider host range, and a higher infectious dose is required to cause disease in humans in most cases. Only 10-100 infectious NoV particles are needed to cause an infection (27). Therefore, a person can

Likelihood of food-Or waterborne transmission	Illness		
	Gastroenteritis	**Hepatitis**	**Other**
Common	Norovirus	Hepatitis A virus	Entrovirus[a]
Occasionally	Enteric adenovirus (Types 40/41)	Hepatitis E virus (waterborne)	
	Rotavirus (Group A-C)		
	Sapovirus		
	Astrovirus		
	Coronovirus		
	Aichivirus		

[a] Entroviruses (e.g. poliovirus) are associated with a range of symptoms including neurological symptoms.

Table 1. Likelihood of foodborne or waterborne viruses, according to type of illness associated with infection

Adapted from Koopmans *et al*, 2004 (63)

become infected via the fecal-oral route by a few viral particles, transmit to close contacts and initiate an outbreak (64). Finally, foodborne viruses have a high level of stability in the environment or on food (29).

The introduction of viruses into the food processing system can occur at any stage from farm to fork. Some documented foodborne viral outbreaks have identified infected food handlers as a major source of contamination (12, 43, 117, 118). In addition, a variety of foods have been implicated in viral outbreaks, which complicates infection control measures (Table 2).

Food category	Norovirus	
	Outbreaks	Cases
Meat	8	441
Poultry	1	67
Eggs	0	0
Dairy	4	4,048
Seafood	3	31
Bread and bakery	12	4,037
Produce	44	3,856
Beverages	8	353
Multi-ingredient foods	175	13554
Other	19	694
Total	274	27,081

Table 2. Food categories associated with NoV outbreaks

Adapted and modified from Greig *et al*, 2007(43)

1.2.1 Noroviruses

Non-bacterial acute gastroenteritis (AGE) was first recognized during the 1940s and 1950s when a group of healthy volunteers were challenged with bacteria-free stool filtrates derived from ill patients. The volunteers demonstrated symptoms of AGE and the causative agent was predicted to be viral (34, 40). With the advent of mammalian tissue culture techniques during the 1960s, no viral agent could be grown from stool samples (25, 57). Finally, with the use of immune electron microscopy (IEM), scientists were able to detect the pathogenic agents (Figure 1) contained in specimens that were sampled from a gastroenteritis outbreak in an elementary school in Norwalk, Ohio (25, 26, 58). These Norwalk viruses and viruses similar in morphological and antigenic characteristics were called "small round structured viruses" (SRSVs) (19), until they were taxonomically assigned to the *Caliciviridae* family (42). Genome sequence of type strains including Snow Mountain virus (125) and Hawaii virus (73) placed them in a new genus termed "*Norwalk-like viruses*" (NLVs), which recently have been named *Norovirus* (NoV).

Human NoV causes self-limiting, species-specific gastroenteritis (27, 111), which can lead to large outbreaks with a considerable economic impact (77). NoV–related outbreaks usually occur in closed settings (46) such as hospitals, nursing homes (38, 39, 91), and cruise ships (52, 124). NoV infects all age groups and can lead to hospitalizations or even death in young children, the elderly or immuno-compromised patients (41, 75). The symptoms of NoV infection include diarrhea, nausea, vomiting, abdominal cramps, fatigue, fever and malaise (60), and the incubation time is estimated between 24 to 48 hrs. The symptoms typically last 1 to 3 days, although symptoms may persist longer among susceptible and immuno-compromised patients. Infected individuals can shed virus up to 3

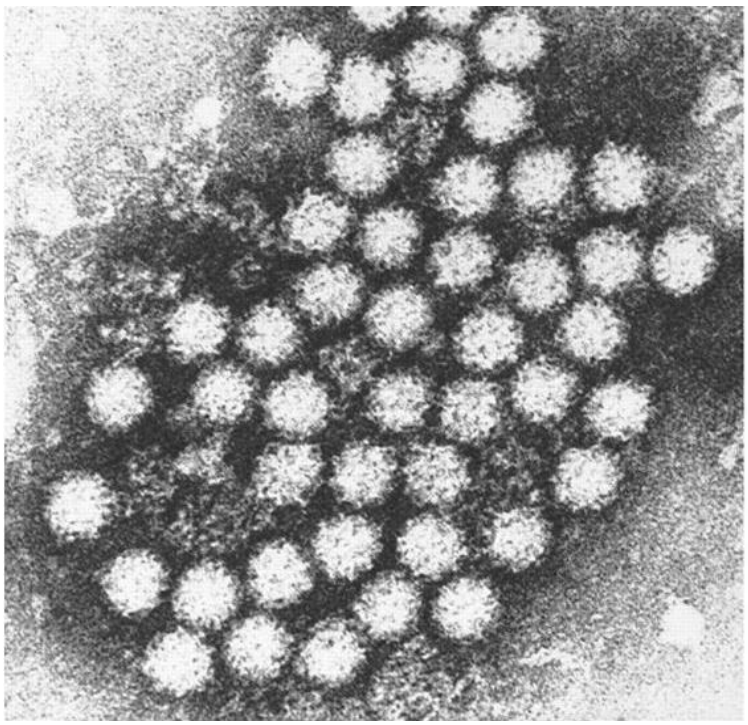

Figure 1. The visualization of Norwalk agent (8FIIa) in stool filtrate

An aggregate observed after incubation of Norwalk (8FIIa) stool filtrate with a 1:5 dilution of serum containing specific antibodies and negative staining for electron microscopy.

Adapted from Kapikian *et al,* 2000 (57)

weeks, although PCR-amplification of NoV in stool has shown a longer period of 100 days for virus shedding in children (60, 62).

NoVs are non-enveloped, single-stranded, positive-sense RNA viruses surrounded by an icosahedral capsid, and are 27-34 nm in diameter. The NoV genome is approximately 7.5 to 7.7 kb in length and is composed of three open reading frames (ORFs) (Figure 2). Interestingly, recent investigations have identified that the murine norovirus (MNV) genome contains an additional ORF (ORF 4) overlapping ORF 2 (78, 114) but the role of this ORF has yet to be elucidated.

ORF 1 (the 5' proximal region) encodes for a 200-KDa polyprotein that is processed into six non-structural proteins including p48, NTPase, p22, VPg, protease $3CL^{pro}$, and RNA-dependent RNA polymerase (RdRp) (45). ORF 2 and ORF 3 encode for the major and minor capsid proteins, respectively. Every norovirus virion contains 180 copies (90 dimers) of major capsid protein or VP1, assembling into a T=3 icosahedral symmetry. VP1 is divided into an S (shell) and a P (protruding) domain that is further subdivided into P1 and P2. The subdomain P2 is of considerable interest as it is the most hypervariable region of the genome and has been implicated in antigenicity and HBGA (histo-blood group antigens) binding (16, 110). The HBGAs are found on the surface of the red blood cells and the mucosal epithelium (112). The minor capsid protein or VP2 is present at 1-2 copies per virion, is presumed to stabilize VP1 (8) and have a role in encapsidation of viral genomes (45).

NoVs are classified into five genogroups (GI-GV) (Figure 3) (132). The genogroups which are involved in human disease are GI, GII, and GIV. Each genogroup is further subdivided into more genetic clusters or genotypes. The classification is based on similarity

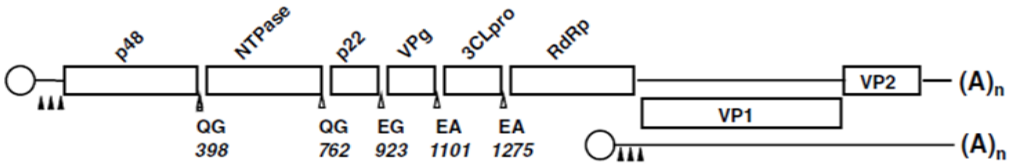
p48
NTPase
p22
VPg
3CLpro
RdRp
VP2
(A)n
VP1
(A)n
QG
398
QG
762
EG
923
EA
1101
EA
1275

Figure 2. Diagram of the NoV genome organization (Norwalk strain)

Non-structural proteins in ORF1 are labeled 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. Adapted from Hardy *et al.* (2005) (45)

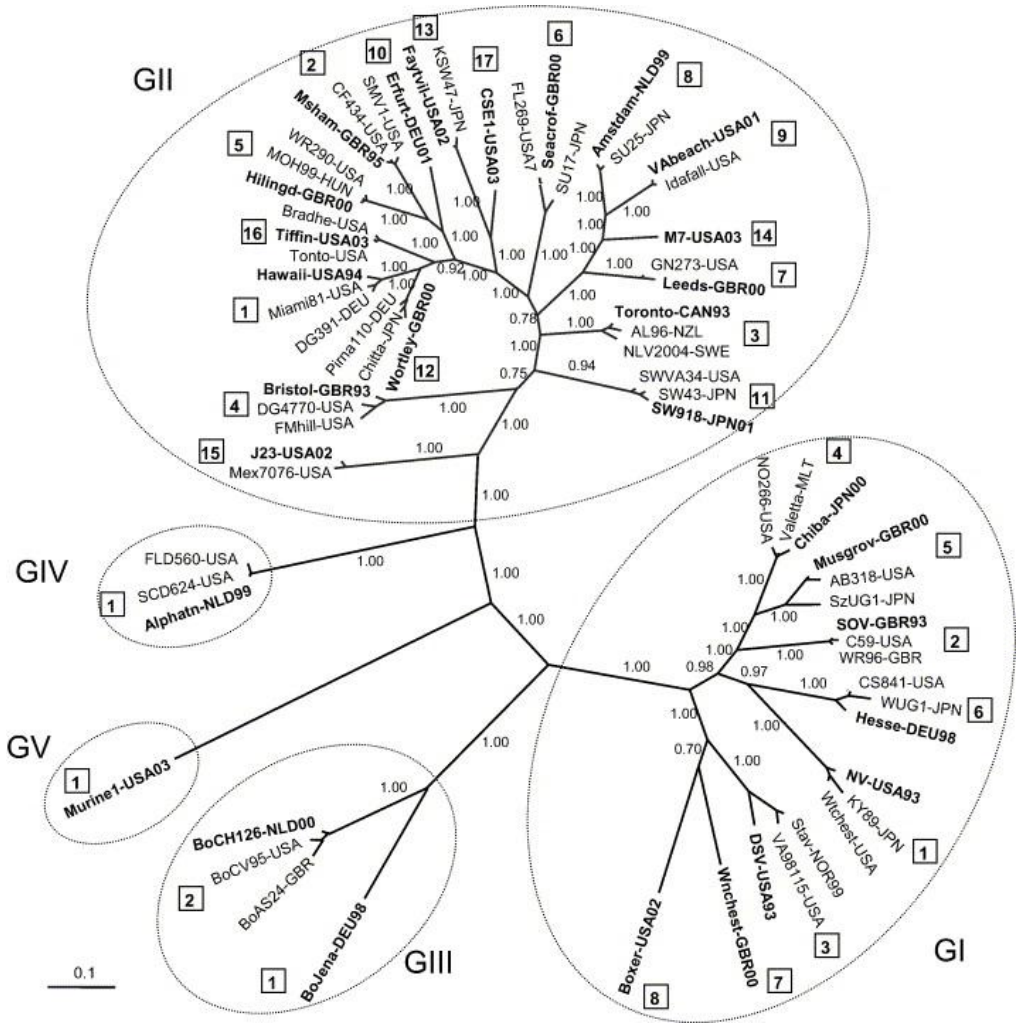
GII
GIV
GV
GIII
GI
0.1
Msham-GBR95
SMV1-USA
CF434-USA
Erfurt-DEU01
Faytvil-USA02
KSW47-JPN
CSE1-USA03
FL269-USA7
Seacrof-GBR00
SU17-JPN
Amstdam-NLD99
SU25-JPN
VAbeach-USA01
Idafall-USA
M7-USA03
GN273-USA
Leeds-GBR00
Toronto-CAN93
AL96-NZL
NLV2004-SWE
SWVA34-USA
SW43-JPN
SW918-JPN01
WR290-USA
MOH99-HUN
Hillingd-GBR00
Bradhe-USA
Tiffin-USA03
Tonto-USA
Hawaii-USA94
Miami81-USA
DG391-DEU
Pima110-DEU
Chitta-JPN
Wortley-GBR00
Bristol-GBR93
DG4770-USA
FMhill-USA
J23-USA02
Mex7076-USA
FLD560-USA
SCD624-USA
Alphatn-NLD99
Murine1-USA03
BoCH126-NLD00
BoCV95-USA
BoAS24-GBR
BoJena-DEU98
NO266-USA
Valetta-MLT
Chiba-JPN00
Musgrov-GBR00
AB318-USA
SzUG1-JPN
SOV-GBR93
C59-USA
WR96-GBR
CS841-USA
WUG1-JPN
Hesse-DEU98
NV-USA93
KY89-JPN
Wtchest-USA
Stav-NOR99
VA98115-USA
DSV-USA93
Wnchest-GBR00
Boxer-USA02

Figure 3. Phylogenetic relationships of norovirus

Noroviruses are classified into five genogroups (GI-GV) which can be further divided into genotypes (e.g. GII.4 represents genogroup II, genotype 4 viruses). Viruses in genogroup I and IV have been found in humans, genogroup II viruses cause disease in both humans and swine, genogroup III NoVs are of bovine origin and genogroup V consists of the newly discovered murine norovirus. Adapted from Zheng *et al*. (2006) (132)

in amino acid sequence of VP1 (123, 132). GI and GII contain 8 and 19 genotypes (55, 132), respectively, of which GII.4 accounts for 80% of all NoV outbreaks throughout the world (32). The viruses in the GIV genogroup are rarely reported in outbreaks.

The susceptibility of individuals to NoV infection is somewhat host-specific, which was first discovered in volunteer studies in 1997 (94). Some individuals are resistant to NoV infection and it has been shown that the infection is highly dependent on the presence of HBGA receptor(s) on the gastrointestinal and oral mucosa. This is noteworthy since over 80% of American and European populations are secretors, indicating that they express the appropriate HBGA receptor(s) on their gastric mucosa (74), whereas the remaining are non-secretors and are resistant to infection with many strains of human NoV. So far, eight patterns of receptor-binding have been suggested (16). As described above, the VP1 capsid protein contains two subdomains P1 and P2, P2 being the least conserved and constitutes the outer surface of the NoV capsid (111). It has been suggested that a binding-pocket in P2 subdomain is involved in receptor-binding which can be disrupted by amino acid substitution (18, 110).

1.3 Transmission of human norovirus

The infectious virus is transmitted through the fecal-oral route, by ingestion of contaminated food or water, or person-to-person contact. Shellfish and ready-to-eat foods are the most common sources of foodborne NoV outbreaks (7, 20, 89, 127). Lettuce and other fresh produce have also been linked to large NoV outbreaks (31, 47, 76, 83). Environmental surfaces, soil and biosolids in the field, and symptomatic or asymptomatic food workers have been associated with NoV transmission (Figure 4). Of 348 NoV outbreaks that were reported to the Centre of Disease Control (CDC) in the USA between January 1996 and November 2000, 39% of outbreaks were associated with foodborne transmission, 12% were person-to-person contact, 3% were waterborne and 46% were resulted from unknown transmission routes (86).

1.3.1 Transmission via food handlers

Food handlers have been implicated in the majority of NoV outbreaks (12, 43, 117-119). Food handlers can contaminate ready-to-eat foods such as sandwiches, salads, bakery products, fruits and vegetables (69, 105). In 2006, in Lansing, Michigan, food workers were found to have contributed to ~ 800 NoV infections in 3 large outbreaks. Greig *et al.*,2007, reviewed a total of 816 foodborne outbreaks between 1927 and 2006 where food handlers were involved (43). Out of the 816 total outbreaks, 491 outbreaks were caused by foodborne viruses with human NoV being the most dominant viral agent in 55.8% of the outbreaks from the last 2 decades. An infected food handler can shed virus before the onset of the symptoms and viral shedding can last for an extended time after symptoms have ceased.

Therefore, it has been suggested that an infected food handler should be excluded from working for at least 24 to 48 hrs after the symptoms have resolved.

1.3.2 Transmission via water

Enteric viruses can enter the water supply via soil, biosolids, stool and vomitus. Contaminated water can be found in multiple resources and used for any application. Some examples include: municipal water (23), well water (28), surface water (*e.g.*, ponds, lakes, rivers, and streams) (43), swimming pool water (117) and commercial ice (30) or irrigation water, produce wash water, and shellfish harvesting water in a food processing system.

Mineral water has never been reported in any NoV outbreaks except for one incident of bottled mineral water which was suspected to be the cause of a NoV outbreak in a German recovery home (10). In addition, in a study by Beuret *et al.* in 2002, the genome of NoV was detected in 33% of the bottled-water samples tested within a year of study (10). This indicates the potential for bottled water to transmit NoV.

One of the most recognized sources of NoV infection are contaminated shellfish, which can bio-accumulate any virus in their growth water. This concentration of enteric viruses in the digestive glands and gills (92) could explain why shellfish account for many international NoV outbreaks (7, 70). Oysters harvested from NoV-contaminated water are likely to cause illness, since they are frequently eaten raw (97, 108). Depuration is used to reduce contamination in shellfish, this involves placing harvested shellfish in clean salt water; however, depuration appears to be less effective at removing viruses than bacteria (103).

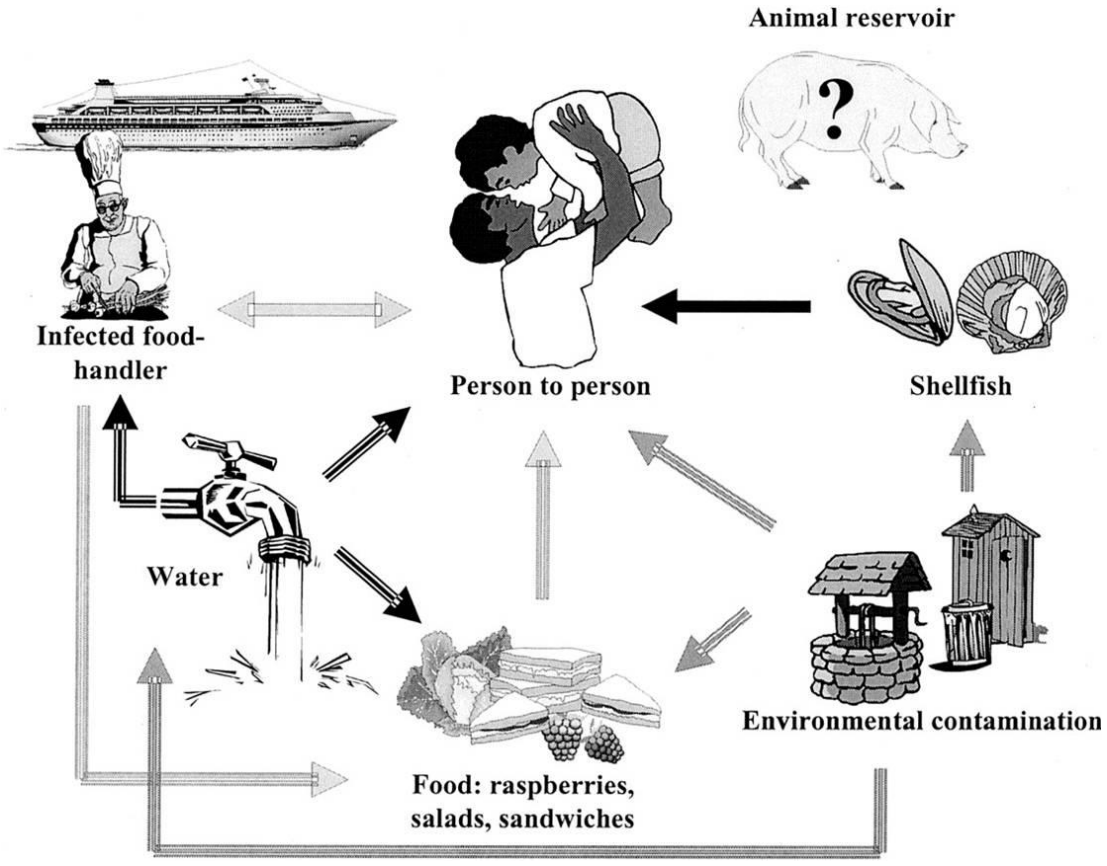
Animal reservoir
?
Infected food-handler
Person to person
Shellfish
Water
Environmental contamination
Food: raspberries, salads, sandwiches

Figure 4. Modes of transmission of norovirus

Different routes of norovirus transmission include person-to-person contact, infected food handlers, contaminated irrigation water, contaminated ready-to-eat foods (e.g. raspberries, salad, sandwiches etc), and contaminated shellfish. Zoonotic transmission is possible but has not been confirmed.

1.3.3 Transmission at the farm level

There are multiple potential sources for crop contamination along the farm-to-fork continuum including: sewage-contaminated irrigation water or fertilizer during production, and harvesting contaminated post-harvest contact surfaces (22) or infected food handlers (12, 43, 117-119). The increasing incidence of NoV outbreaks associated with the consumption of salads and fresh produce raises concerns that agricultural lands might be a source of contamination. Organic fertilizers or animal manure are used for agricultural purposes and could introduce enteric viruses to the soil or growing plants (15, 82, 129). In addition, it has been previously reported that the strain GII.4 (human NoV) was detected in cattle and swine feces (82). This indicates that animal manure or biosolids are a potential route of contamination for growing plants or irrigation water. Despite the presence of nearly 150 known enteric pathogens in untreated waste (36, 51), wastewater treatment has failed to eliminate enteric viruses after secondary and tertiary sewage treatment (116). Therefore, viruses present in sewage-contaminated irrigation water or in fertilizers and biosolids could contaminate the soil and growing crops, or contaminated water could increase the chance of crop contamination. Given the fact that only a few infectious NoV particles are required to cause disease (27, 113) and that fresh produce distributed to retail channels with minimal disinfection, any virus introduced on a farm may have a public health impact.

It is important to understand the dynamics of microbial survival on fresh produce, to determine the public health risks related to naturally-occurring or intentional contamination in the production environment. This will inform new risk assessments with accurate descriptions of the stability of microorganisms in the environment.

1.4 Environmental stability of norovirus

In general, viruses are inert particles outside of their host and must maintain all of their components in order to retain their infectivity. Whether or not viruses can be transmitted from environment to susceptible hosts and cause disease relies on the degree of virus stability. The terms “stability”, “persistence” and “survival” are commonly used in literature of environmental virology which refer to the natural capacity of viruses to resist various environmental scenarios (98).

Direct studies on the stability and persistence of human NoV are not possible since cell culture systems or small animal models are not available to determine the degree of viral infectivity (29). Therefore, the use of a surrogate is essential and currently two viruses, MNV and feline calicivirus (FCV) belonging to the family *Caliciviridae*, have been used by researchers to approximate the stability and persistence of human NoV on various matrices. Previous studies have suggested that MNV is a better surrogate for the environmental behaviour of human NoV than FCV (4, 17).

Several studies investigating the stability of enteric viruses in environmental settings have used common methodologies (17, 66, 81). In most cases, known amount of infectious virus is added directly or indirectly to an environment which has the potential to transmit enteric viruses (such as water, soil, lettuce, and other matrices). The spiked samples are stored under designated conditions for specified time periods. The viruses are then extracted from the matrices and the fate of the virus is determined. The extraction and detection techniques can differ. Finally, the decay in virus infectivity is determined through cell culture assays and statistical analysis.

Virus stability is influenced by a variety of environmental conditions. In addition, the presence of an envelope is a significant biological factor that reduces the persistence of viruses when they are dried. Non-enveloped viruses, such as NoV, have much higher resistance in the environment and are therefore spread more easily by indirect person-to-person contact (50). Factors which affect virus stability on surfaces include relative humidity (RH), temperature, solar radiation, ultra violet (UV), type of the surface, pH, adsorption state and organic matter (121). NoV persists at a wide range of temperatures from refrigeration to freezing. Mattison *et al*. (2007) indicated that low temperature (4°C) favours the stability of FCV on both food and stainless steel surfaces (81). Baert *et al.* (2008) found no reduction in MNV infectivity on frozen onions and spinach after 6 months storage. Lamhoujeb *et al*. (2009) could detect infectious NoV on surfaces up to 56 days at 7°C (68). The type of surface also plays an important role in determining virus stability. Studies on metal, fruit and vegetable surfaces suggest that NoV may have greater stability on coarse surfaces than on smooth surfaces (81), however, some specific surfaces may represent virucidal properties and contribute to the inactivation of viruses. Adsorption of viruses to the surfaces (such as metal equipment, vegetable and fruits, soil, and water sediments) is another critical factor and involves electrostatic and hydrophobic interactions (122). The effect of pH is less dominant with regards to persistence or inactivation in the environment since enteric viruses are resistant to acidic conditions in the GI tract. It has been reported that NoV remains infectious after 3 hrs of exposure to pH 2.7 in volunteer studies in 1972 (25). A recent study by Horm *et al*., 2011, has shown that NoV surrogates remain stable in juice blends (pH: 3.34 to 3.83) at refrigerating temperature for 21 days (48).

Stability in soil depends on the type of the soil, state of adsorption, presence of microorganisms, moisture content, soil temperature, rainfall and solar radiation (100). Hurst *et al.* (1980) suggested that both temperature and virus adsorption to soil can impact virus stability.

Overall, data on the persistence and stability of human NoV are inadequate and therefore it is difficult to determine the chances of virus transmission from the environment to the food supply or to the human population.

1.5 Detection of norovirus in food and environmental samples

In the past, human NoV was detected in stool suspensions by electron microscopy (EM) techniques (3), which is a relatively insensitive method, requiring over 10^6 particles per milliliter. This method is still sometimes used for the detection of NoV in stool samples but is not reliable for detection in food and environmental samples, due to the lower concentrations of NoV particles. Sequencing of the genome of NoV has permitted the detection of these viruses via molecular techniques such as real-time and conventional reverse transcription polymerase chain reaction (RT-PCR) assays. However, due to variability in the genomes of NoV strains, one set of primers cannot detect all NoV and therefore many RT-PCR primer sets have been developed that target different regions of the NoV genome. The regions coding for the RdRp and the ORF1/ORF2 junction on the genome are highly conserved and are mostly used for the design of primers (2, 54, 59, 67).

Although genomic methods have facilitated the detection of NoV in food, water and environmental matrices, virus isolation and nucleic acid extraction are challenging. As described previously, the level of NoV contamination is usually low within a sample and a large volume of food (25-100 g) or water (1-100 L) has to be processed and concentrated to detect the virus. It is worth noting that, unlike bacteria, there is no culture enrichment method available for viruses. Methods to concentrate NoV from environmental matrices include adsorption-elution, PEG precipitation, and ultrafiltration (53, 61, 107). A recent publication by Shinohara *et al.,* (2011), suggested a new technique for concentration of NoV from water samples (106). The method uses minute particles of amorphous calcium phosphate (ACP) to capture NoV particles in water. The adsorption-elution method with the use of 1MDS filters has also been successful in recovery and detection of enteric viruses

from water, lettuce and fruit (9, 14). The problem with concentration methods is that inhibitors, which may be present in food and environmental matrices can be co-concentrated, interfere with enzymes involved in conventional or real-time RT-PCR, and lead to false negative results. In addition, methods that detect NoV based on viral nucleic acid cannot confirm infectivity.

1.6 Hypothesis and Objectives

Hypothesis: Infectious NoV persists on various environments long enough to pose risks to food and consumer safety.

Objective: The objective of this project was to determine the persistence of MNV in food processing and agriculturally-relevant environments. The long-term goal is to provide data for modeling the potential impact of contamination in the Canadian food supply.

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2 MATERIALS AND METHODS

2.1 Cell culture and virus

The cultures of murine microglial cell line BV-2 were kindly provided by Dr. Wobus (University of Michigan). The cells were grown in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen, Burlington, ON) supplemented with 10% fetal bovine serum (FBS), 0.1 mg/ml L-glutamine, and 0.1 mg/ml penicillin-streptomycin. The cells were incubated at 37 ºC with 5% CO_2 (21).

Murine norovirus 1 clone CW3 (MNV-1) was kindly provided by Dr. Virgin (Washington University School of Medicine). MNV-1 was propagated in BV-2 cells as previously described (4, 21) with minor modifications. Briefly, cells were seeded in 175-cm^2 tissue culture flasks and incubated for 24-48 hours for the formation of a confluent monolayer. The medium was then completely removed and cells were washed twice with phosphate buffered saline (PBS). The infection was carried out at a multiplicity of infection (MOI) of 0.05 and cells were covered with 5 ml of serum-free DMEM and incubated for 1 hour at 37 ºC with 5% CO_2. Following the 1 hour adsorption, 15 ml of serum-free medium was added to the cells and they were incubated for 48 hr. Finally, cell lysates containing MNV-1 were pooled followed by 3 cycles of freezing and thawing at -80 ºC and room temperature, and stored in 1 ml aliquots at -80 ºC until use.

2.2 Matrices

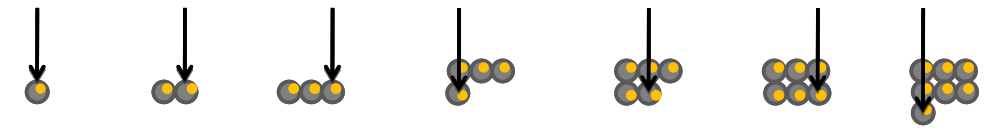
For virus stability experiments, iceberg lettuce and bottled water were purchased from a local store, stainless steel disks were kindly provided by Dr. S. Sattar (University of Ottawa) and sandy soil and loamy soil were obtained from Agriculture and Agri-Food Canada farms in Summerland, BC (Dr. Pascal Delaquis) and Kentville, NS (Dr. Greg Bezanson), respectively.

2.3 Stability experiments

A virus stock was prepared in a soil load to mimic fecal matter as described by Sattar *et al.* (101). Briefly, the virus stock was consisted of MNV, 5% Trypton, 5% bovine serum albumin (BSA), and 0.4% mucin. The inoculum was divided into approximately 10 µl and 100 µl aliquots and stored at -80ºC until needed for the days of inoculation.

Stainless steel disks (1cm diameter) were washed with sterile water, dried at room temperature and autoclaved (81). One disk was inoculated every 7 days with 10 µl of the virus preparation containing 2 x 10^5 plaque forming units (PFU) for 42 days (6 times, 6 disks). The disks were incubated at room temperature in a 12-well plate, until "day 0" (last inoculation performed on the 42^{nd} day of the experiment) where an additional 7^{th} disk was inoculated with the same quantity of the virus and dried for 20 min (Figure 5). Then all samples were recovered from each of the seven disks by pipetting (25 times) in 1 ml of Earle's Balanced Salt Solution (EBSS) (SIGMA, St. Louis, MO).

Inner leaves of iceberg lettuce were cut into 2 x 2 cm pieces, rinsed with autoclaved water, allowed to dry under laminar airflow for 20 min and sterilized with UV light on each side for 1 min. One piece of lettuce was inoculated every 3 days with 10 µl of the virus



DAY 42 35 28 21 14 7 0

START POINT END

* Recover all samples

Figure 5. Inoculation of disks

One disk was spiked with 10 μl of MNV-1 suspension at 7-day intervals for 42 days at room temperature. All samples were recovered on “Day 0”.

preparation, containing 2 x 10^5 PFU, for 15 days (5 times, 5 pieces of lettuce). The lettuce was incubated at room temperature in a 12-well plate, until "day 0" where an additional 6^{th} sample was inoculated with the same quantity of the virus and dried for 20 min. Then all samples were recovered from each of the six samples by pipetting (25 times) in 1 ml of EBSS.

One gram of soil was inoculated every 7 days with 100 µl of virus, suspension containing 8 x 10^5 PFU for 42 days. The soil was incubated in screw cap tubes (Ultident Scientific, St. Laurent, QC) and each tube was wrapped in parafilm to prevent moisture evaporation from the soil. On "day 0" the viral inoculum was incubated for 20 min and then virus was eluted from all samples with 5 ml of EBSS. The soil was removed by centrifugation at 4000 rpm for 15 min. The supernatant was filtered sequentially through 0.45 µm filters and 0.22 µm filters (Millipore, Bedford, MA). The filtrates were stored in 100 ml aliquots prior to plaque assay and RNA extraction.

One 500 ml bottle of water was inoculated every 7 days with 100 µl of virus suspension containing 2 x 10^6 PFU for 42 days. Bottles of water were stored at room temperature and viruses were recovered from all samples, plus a t=0 interval, using an adsorption/elution method (section 2.4).

2.4 Virus concentration method

The Absorption/Elution method, previously validated in the laboratory for recovering enteric viruses from large samples of water and lettuce (14, 80), was used. Five hundred ml of water were filtered through a positively charged 1MDS membrane (Kinecor, Ottawa, ON) to absorb the suspended viral particles. Then each membrane was placed into a container holding 10 ml of Tryptose Phosphate Broth with 6% Glycine (TPBG) at Ph 9.0 and rotated on a shaker for 30 min at room temperature to resuspend the viruses from the charged membranes. The solution containing the MNV particles was adjusted to pH 7.0-7.6 with 1N HCl and 3.5 ml was transferred to the Amicon ultra centrifugal filter with 100 kDa membranes (Millipore, Billerica, MA) for further concentration. The Amicon filters were centrifuged at 3750 rpm for 10 min, and then the absorbed viruses were washed by pipetting from both membranes with 250 µl of PBS. The Amicon step was repeated 2 times for each sample to reach the final volume of 500 µl. The concentrate was divided to 240 µl for plaque assay (section 2.5.1) and 140 µl for RNA extraction (section 2.5.2).

2.5 MNV-1 Detection Methods

2.5.1 MNV Plaque assay

The MNV plaque assay previously described by Cox *et al.,* (2009), was performed with some modifications (21). Prior to infection, murine BV-2 cells were seeded into 12-well plates at density of 5 x 10^5 cells in 2 ml growth medium (DMEM) per well and were incubated for 24 hr at 37 ºC with 5% CO_2. The growth medium was then removed and the cells were washed twice with PBS. The solutions containing eluted MNV particles from each of the matrices were diluted in EBSS and 0.1 ml of each dilution was placed in each of the 3 wells of a plate. The plates were incubated at 37 ºC with 5% CO_2 for 1 hour and rocked gently every 10 min. Following the incubation, the virus inocula were removed and cells were washed with PBS. All the wells were overlaid with 0.7% agarose and reincubated for 48 hrs. The cells were fixed with paraformaldehyde and stained with 0.1% crystal violet. The plaques were counted and multiplied by the dilution to obtain virus titre in PFU/ml.

2.5.2 RNA Extraction

RNA was extracted from 140 µl of concentrated MNV eluate using a QIAamp viral RNA kit (Qiagen, Maryland, USA) according to manufacturer's instructions. The MNV solution was denatured with a denaturing buffer "AVL" containing carrier RNA, and incubated at room temperature for 10 min. For efficient binding, absolute ethanol was added to the sample and inverted for 15 sec. The solution was transferred to a silica gel membrane for RNA binding and centrifuged at 8,000 rpm for 1 min. The membrane was washed twice, first with "AW1" buffer and secondly with "AW2". Finally, 60 µl of the elution buffer "AVE" was added to the membrane, incubated for 1 min at room temperature and centrifuged at 8,000 rpm for 1 min. The extracted RNA was stored at -80 ºC until needed.

2.5.3 Conventional RT-PCR

Conventional reverse-transcription PCR (RT-PCR) was performed using a One-step RT-PCR kit (Qiagen) on a Mastercycler PCR machine (Eppendorf, Hamburg, Germany). The PCR reaction consisted of 5 µl of purified RNA, 1X Qiagen One-Step RT-PCR buffer, 400 µM dNTP mix, 0.6 µM of MNV-1f and MNV-1r primers (Table 3), 1 µl Qiagen One-step RT-PCR enzyme mix and 4 units of RNase Inhibitor in a final volume of 25 µl. Cycling conditions were 30 min at 50 ºC, 15 min at 95 ºC, and 40 cycles of 1 min at 94 ºC, 45 sec at 60 ºC and 1 min at 72 ºC followed by a 5 min final extension at 72 ºC. The PCR products were run on a 2% agarose gel stained with 0.5 X SYBR Safe (Invitrogen) at 135 V for 60 min. All RT-PCR products were stored at -20 ºC.

2.5.4 Real-time RT-PCR

Real-time RT-PCR was performed using the Brilliant II one step RT-PCR kit (Stratagene, La Jolla, CA) on a Stratagene Mx3005P instrument. The assay was developed by Baert *et al.* (5). Briefly, 25 µl of the reaction contained 2 µl of the purified RNA, 1X CORE RT-PCR buffer, 2mM of MgCl2, 200 nM dNTPs, 200 nM of each primers (Fw-ORF1/ORF2, Rv-ORF1/ORF2), 200 nM probe (Table 4), 50 nM Reference dye (ROX) diluted (1:500) in RNase/DNase-free water (Invitrogen), 2.5 U SureStart *Taq* DNA polymerase and 2.5 U Stratascript reverse transcriptase. The cycling conditions include 50ºC for 30 min for reverse transcription, then initial denaturation and activation of *Taq* polymerase at 95ºC for 10 min, followed by 40 cycles of 15 s at 95ºC and 1 min at 60ºC.

2.5.4.1 Standard Curve for real-time RT-PCR

The standard curve was generated from 10-fold serial dilutions of an RNA transcript generated from a cDNA copy of the ORF1/2 junction of MNV (88). RNA template was extracted using QIAamp viral RNA kit and was reverse transcribed with QIAGEN One-Step RT-PCR kit using MNV primers to generate a cDNA copy of ORF1/2 junction of MNV. The insert was cloned into pCR2.1 vector with TOPO TA Cloning kit (Invitrogen) according to manufacturer's instructions and the positive clones were screened with PCR. The plasmid was isolated using a Qiagen miniprep kit (Qiagen), digested and transcribed with MEGAscript-High Yield transcription kit (Ambion, Austin, TX). The RNA product was quantified by Nanodrop ND-1000 spectrophotometer (Thermo Scientific, Wilmington, DE). The 10-fold dilutions of the RNA transcript were used as a standard curve in MNV Real-time RT-PCR assay.

	Primer/Probe	Sequence (5' to 3')	Reference
Conventional RT-PCR			
MNV-1	MNV-1f	CCG AGA CCA CCA AGA CTG GAG	Anu Shukla
	MNV-1r	GAC GCG AAC CAG GAG ACA AAC TC	Anu Shukla
Real-time RT-PCR			
MNV-1	Fw-ORF1/ORF2	CAC GCC ACC GAT CTG TTC TG	(5)
	Rv-ORF1/ORF2	GCG CTG CGC CAT CAC TC	(5)
	MGB-ORF1/ORF2	6FAM-CGC TTT GGA ACA ATG-MGBNFQ	(5)
FCV	SH-FCV3-Q-A	GAC ACC TCC GAC GAG TTA TGC	(80)
	SH-FCV3-P	CCG GGT GGG ACT GAG TGG	(80)
	SH-FCV3-Q-1	CY5-CGC CTT ACG GAT ATG AGC AGC CAC ATT AAC-BHQ	(80)

Table 3. Primers and probes used in RT-PCR assays for the detection of MNV-1 and FCV

2.6 Experimental plot preparation and crop planting

The field was located at the Atlantic Food and Horticulture Research Centre, in Kentville, NS.

The crop planting was conducted by a research team in Kentville under the supervision of Dr. Greg Bezanson. Briefly, greenhouse-grown transplants were treated for 3 weeks in the greenhouse followed by hardening for one week prior to planting. Manual weeding, chemical fertilizer and pesticides were used as needed.

The plot used for this trial was 4x4 m in size. Seedlings for 240 heads of romaine lettuce were initially double-planted in 6 rows 80 cm apart from each other (Figure 6) and were thinned to 20 plants per row or 120 plants in the entire plot. Dripline irrigation was used to water the plants.

The trial commenced on July 26th 2010 and lasted 14 days for lettuce and 28 days for soil. The first lettuce and soil specimens were harvested immediately after inoculation and shipped to laboratory for further analysis. Samples were received from July 27th to August 10th 2010 at 7-day intervals. The first samples (day 0) consisted of 15 inoculated lettuce, 3 inoculated soil and the same numbers of control samples (uninoculated).

2.7 Farm inoculation

The plot was inoculated 5 weeks after the crops were established on July 26th 2010. The irrigation cocktail consisted of *E. coli* O157:H7, *Bacillus atrophaeus* spores, *Eimeria papillata* oocytes, and MNV-1. The plot was inoculated with a watering can.

2.8 Preparation of norovirus inocula

A large volume of highly concentrated stock of MNV was made as previously described (21). No concentration step was performed. Ninety ml of a stock at $1.3x10^9$ PFU/ml was sent to the farm. The field required 2 litres of the cocktail per row (12 litres in total) consisting of $1x10^7$ PFU/ml of MNV-1.

2.9 Sampling strategy

Fifteen lettuce samples were taken every 7 days for 14 days. Each lettuce head was split into 4 sections and sent to research laboratories for further analysis.

Three soil cores (10 cm diameter, 5 cm depth) were taken randomly within the rows of lettuce every 7 days for 28 days. Control samples of lettuce and soil (non-inoculated) were sent together with the samples on the first day of inoculation. The plot contained sandy soil and was similar to the samples tested in the laboratory experiments described in Section 2.3.

2.10 Sample preparation and processing

MNV persistence was determined for lettuce and soil samples from the farm in Kentville, NS. All samples were sent from the farm in the afternoon of the sampling day in coolers and received the next day, when they were processed.

Twenty five grams of each lettuce sample was transferred into a sterile stomacher bag. Each lettuce sample (25 g) was spiked with 50 µl of 10^6 PFU/ml FCV as an internal control (80). Then 225 ml of 1 x PBS was added to each bag and mixed by

manual agitation. The buffer containing the pathogens was passed through 0.45 μm filters and then concentrated with 1MDS filters and Microcon membranes to 10 ml and 500 μl, respectively. The filtrates were kept frozen at -80 ºC until analysis.

Each sampling bag containing soil weighed up to 100 g, from which 1 g was placed into a centrifuge tube and inoculated with 50 μl of 10^6 PFU/ml of FCV. The recovery procedure from soil is described in Section 2.3.


80 cm

Figure 6. Illustration of the Romain lettuce plot, in Kentville, NS

120 lettuce plants were grown in 6 rows, 80 cm apart in a 4 x 4m plot.
The picture was kindly provided by Dr. Greg Bezanson

2.11 Detection Methodology

MNV RNA was extracted as described in Section 2.5.2 and detected using conventional RT-PCR, as described in Section 2.5.3. The presumptive positives were then confirmed by DNA sequencing of the PCR product (Section 2.11.2). MNV infectivity was tested via MNV plaque assay (Section 2.5.1). Real-time RT-PCR was used to quantify MNV in each sample while FCV was quantified to validate the detection method (see below).

2.11.1 FCV assay and real-time RT-PCR

The FCV reaction contained 2 µl of the purified RNA, 1X CORE RT-PCR buffer, 5mM of MgCl2, 800 µM dNTPs, 300 nM of each primer and 200 nM probe (Table 4), 30 nM Reference dye (ROX) diluted (1:500) in water, 1.25 U SureStart *Taq* DNA polymerase and 2.5 U Stratascript reverse transcriptase. The cycling conditions include 50ºC for 30 min for reverse-transcription then the initial denaturation step and activation of *Taq* polymerase at 95 ºC for 10 min, followed by 40 cycles 95 ºC for 15 s at and 60 ºC for 1 min.

2.11.2 Sequencing of PCR products

Lettuce and soil samples were tested for the presence of the MNV genome with conventional RT-PCR as previously mentioned. The amplicons which were approximately 400 bp in size were purified and sequenced in our laboratory. Two different approaches were taken to purify DNA depending on the presence of a single amplicon or multiple amplicons. A multi-band DNA product was purified by gel extraction and a single-band product was purified by Microcon YM-30 Centrifugal Filter Units (Millipore). The Microcon method involves the addition of DNA sample and 100 µl distilled water to the column provided in the kit and centrifugation at 13,000 x g for 8 min. To elute purified DNA, 30 µl of DNase/RNase free water was added to the column and centrifuged at 3,000 x g for 1 min. The gel extraction method was conducted according to the instructions provided in the QIAquick Gel Extraction kit (QIAGEN). Briefly, the DNA fragment was excised from an agarose gel and placed in 1.5 ml microcentrifuge tube containing QG Buffer and heated at 50 ^{o}C for 10 min. Once the gel slice was thoroughly dissolved, an equal volume of isoproponol was added to the DNA and the mixture was transferred to a QIAquick column for DNA binding and centrifuged at 13,000 x g for 1 min. Then the membrane was washed with PE buffer and centrifuged for another 1 min at the same speed. For high DNA recovery, 30 µl of EB buffer was added to the centre of the column, incubated for 1 min at room temperature and centrifuged at maximum speed for 1 min.

Prior to sequencing, the DNA samples were labelled with fluorophores using the BDT or Big Dye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA) according to manufacturer's instructions. Each forward and reverse sequencing reactions contained 3 to 5 µg of purified DNA and 3 pmol of MNV specific forward or

reverse primers (Table 3). The BDT-labelled sequencing reaction was purified using the Wizard Magnesil Sequencing Reaction Clean-up system (Promega, Madison, WI), and eluted with 30 µl of injection solution (Millipore, Bedford, MA). The sequencing was performed using an ABI 3130X Genetic Analyzer sequencer (Applied Biosystems).

2.6 Statistical Analysis

Linear regression analysis was performed using PRISM4 (GraphPad, San Diego, CA). This approach was taken for Laboratory analysis of MNV persistence on lettuce, stainless steel disks, soil and in water. Error bars represent the standard deviation of the mean from three replicate experiments.

3 Results

3.1 Stage A- laboratory analysis of MNV persistence on lettuce, stainless steel disks, soil and in water

3.1.1 Determination of virus recovery from various matrices

In preparation for field trials, I tested a simple and efficient procedure to study the recovery of MNV from various matrices. The virus was incubated for 20 min at room temperature as described in the Materials and Methods to determine the percent recovery from each matrix. This value provides a starting point (t=0) for comparison of the persistence data without the confounding effects of short term drying, dispersal or method efficiency. The results are shown in Table 4. Seventy percent of the virus inoculum (T_i) could be recovered (T_0) from the lettuce surface and 37% from disks. The percent recoveries were similar from loamy and sandy soil, 32% and 25% respectively. Recovery from water ranged from 2.4% to 4.9% with an average of 3.8% over three independent experiments. The percent recovery from lettuce was highest compared to other matrices and the lowest percent recovery was from water. The percent recovery was determined according to the number of infectious particles obtained (T_0) and initial input (T_i).

	T_i (PFU)	T_0 (PFU)	T_0 / T_i (%)
Disk	2.0×10^5	7.3×10^4	37
Lettuce	2.0×10^5	1.4×10^5	70
Loam	8.0×10^5	2.6×10^5	32
Sand	8.0×10^5	2.0×10^5	25
Water	2.0×10^6	7.8×10^4	3.8

T_i, initial virus titre; $T_{0,}$ virus titre recovered after 20 min of equilibration; T_0/T_i%, percent recovery; PFU, Plaque Forming Units.

Table 4. Recovery of MNV from various matrices after 20 min of equilibration

3.1.2 Persistence of viral infectivity on/in various matrices

Figure 7 shows the inactivation of MNV over 42 days at room temperature on the surface of stainless steel disks. A 2.8 $\log_{10}$ decrease in viral titre was observed after 42 days on a steel surface. The best fit regression line calculation estimates a 1 $\log_{10}$ reduction of MNV on stainless steel occurs approximately every 15 days (D = 14.57).

Infectious MNV persistence on the surface of lettuce leaves for 15 days is presented in Figure 8. Infectious virus was recovered up to day 12 post-inoculation and the regression calculation estimates a 1 $\log_{10}$ reduction after approximately 4 days (D = 4.259).

Two types of soil were used in this persistence study, sandy soil and loamy soil, which differ in a variety of characteristics including moisture content (99). The pH of both types of soil was approximately 6.5, and it has been reported that viruses survive longer in neutral soil (6, 51).The results indicate that there was no significant difference in viral inactivation between the two types of soil. D-values corresponding to a 1 $\log_{10}$ decrease in virus titre were calculated to be approximately 12 days for both sandy and loamy soil (Figure 9) (D = 11.70, and 12.14), respectively.

Figure 10 shows the titre of MNV-1 recovered from water incubated at room temperature for 42 days. Only a 1.6 $\log_{10}$ reduction was observed throughout the 42 day experiment. A 1 $\log_{10}$ reduction was calculated on average every 29 days by regression analysis (D = 29.40).

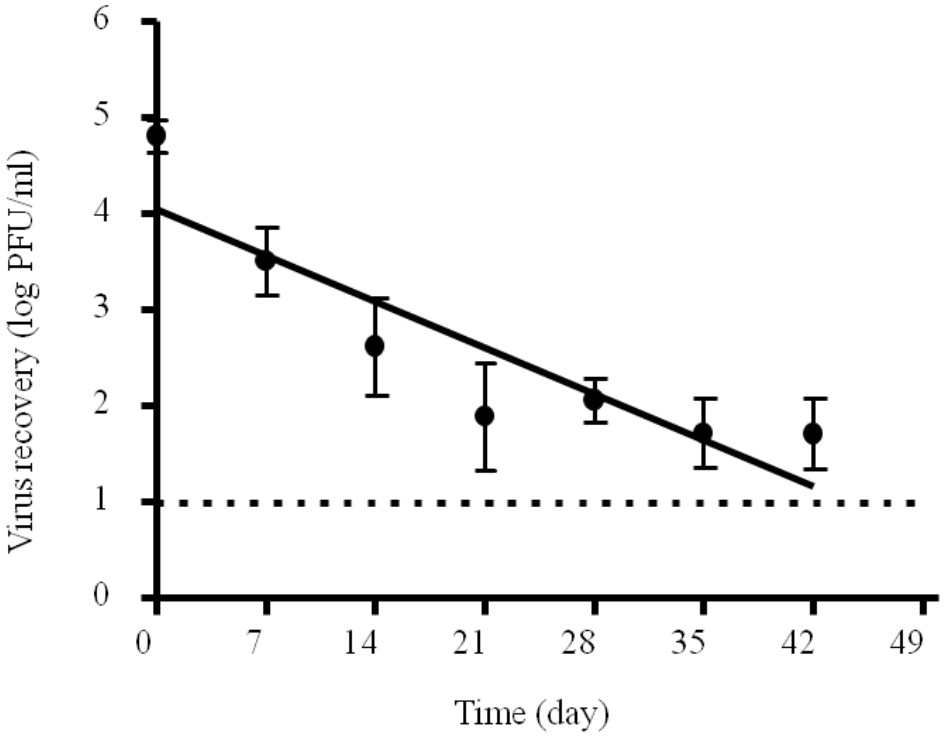

Virus recovery (log PFU/ml)
6
5
4
3
2
1
0
0
7
14
21
28
35
42
49
Time (day)

Figure 7. Survival of MNV-1 on stainless steel disks at room temperature

The dashed line indicates the limit of detection of the plaque assay (10 PFU). The titre at day 0 represents the virus recovered after 20 min of incubation at room temperature. Error bars represent the standard deviations of the mean from three independent experiments.
The regression line represents $Y= 59.07x + 4.054$, $r^2 = 0.7991$
.

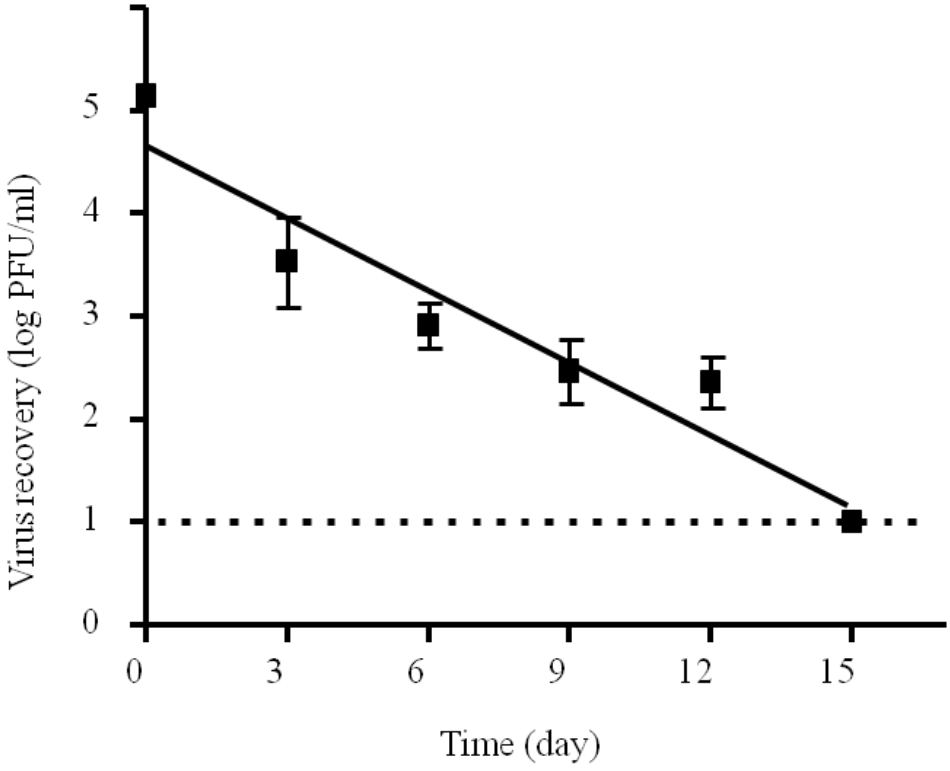

Virus recovery (log PFU/ml)
0
1
2
3
4
5
0
3
6
9
12
15
Time (day)

Figure 8. Survival of MNV-1 on lettuce at room temperature

The dashed line indicates the limit of detection of the plaque assay (10 PFU). The titre at day 0 represents the virus recovered after 20 min of incubation at room temperature. Error bars represent the standard deviations of the mean from three independent experiments. The regression line represents $Y = 19.85x + 4.66$, $r^2 = 0.9135$

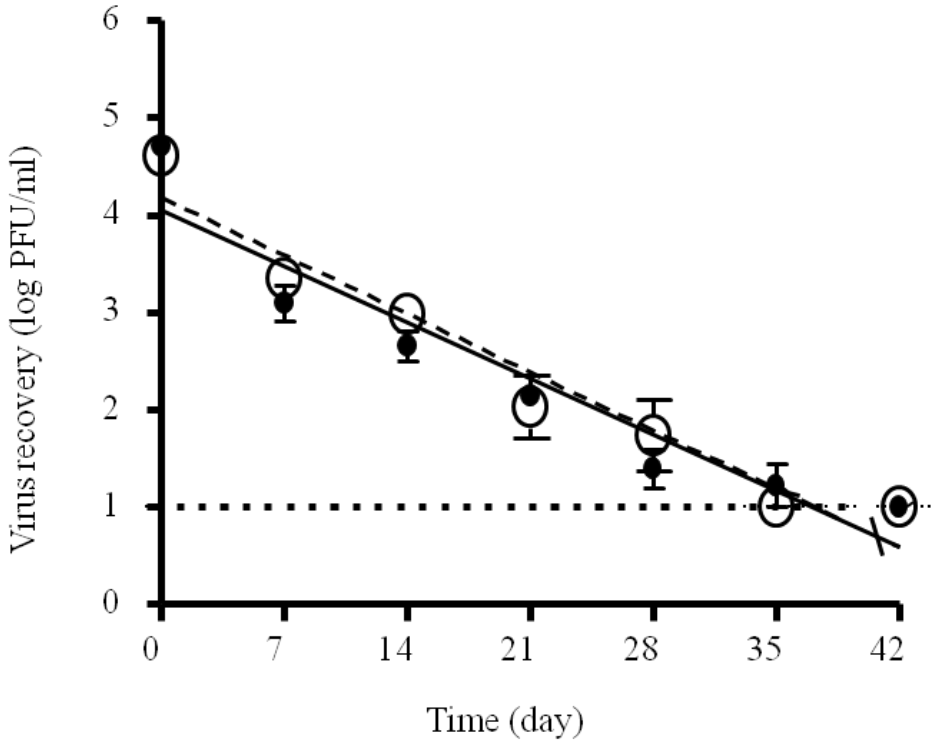

Virus recovery (log PFU/ml)
6
5
4
3
2
1
0
0
7
14
21
28
35
42
Time (day)

Figure 9. Survival of MNV-1 in soil at room temperature

The dashed line indicates the limit of detection of the plaque assay (10 PFU). The titre at day 0 represents the virus recovered after 20 min of incubation at room temperature. Error bars represent the standard deviations of the mean from three independent experiments.
The regression line represents $Y= 49.11x + 4.04$, $r^2 = 0.906$ for loamy soil (solid black circles and solid line), $Y= 48.91x + 4.18$, $r^2 = 0.946$ for sandy soil (empty white circles and dashed line)

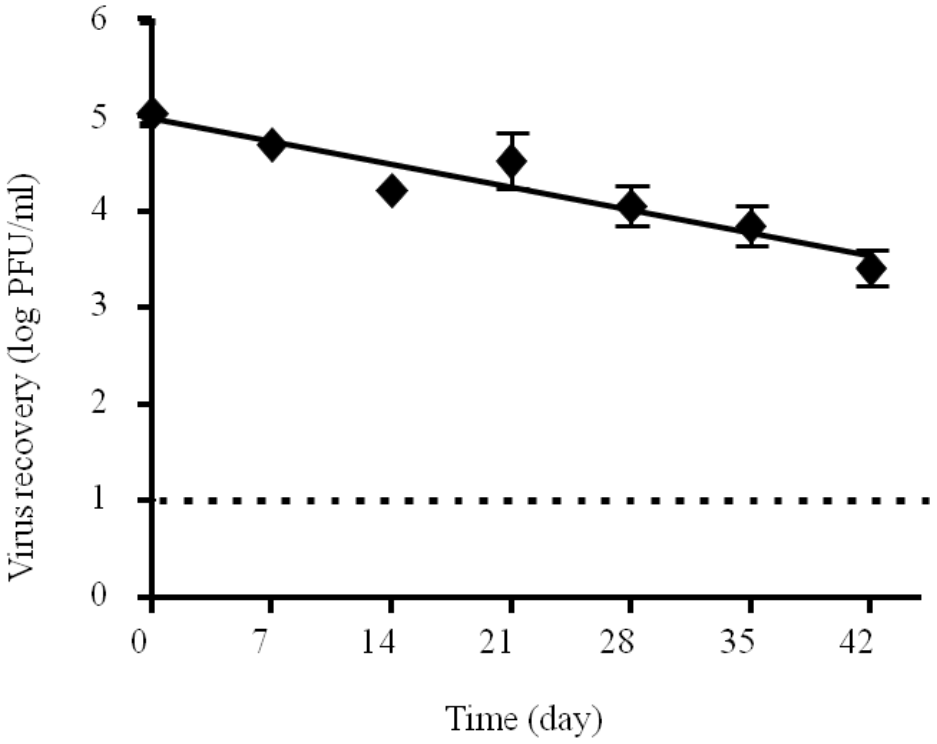

6
5
4
3
2
1
0
Virus recovery (log PFU/ml)
0
7
14
21
28
35
42
Time (day)

Figure 10. Survival of MNV-1 in drinking water at room temperature

The dashed line indicates the limit of detection of the plaque assay (10 PFU). The titre at day 0 represents the virus recovered after 20 min of incubation at room temperature. Error bars represent the standard deviations of the mean from three independent experiments.
The regression line represents $Y = 146.2 + 4.97$, $r^2 = 0.9007$

3.1.3 Persistence of viral RNA on/in various matrices

The MNV genome was detected by real-time RT-PCR and conventional RT-PCR from all matrices tested. The persistence of viral RNA by real-time RT-PCR is shown in Figure 11. The MNV genome persists over time in all matrices. The RNA seemed to be more stable in water, on disks and lettuce, whereas more variability was observed in soil, particularly, in loamy type. These results indicate that there is no correlation between the quantity of infectious MNV and the presence of MNV nucleic acids in/on all matrices tested.

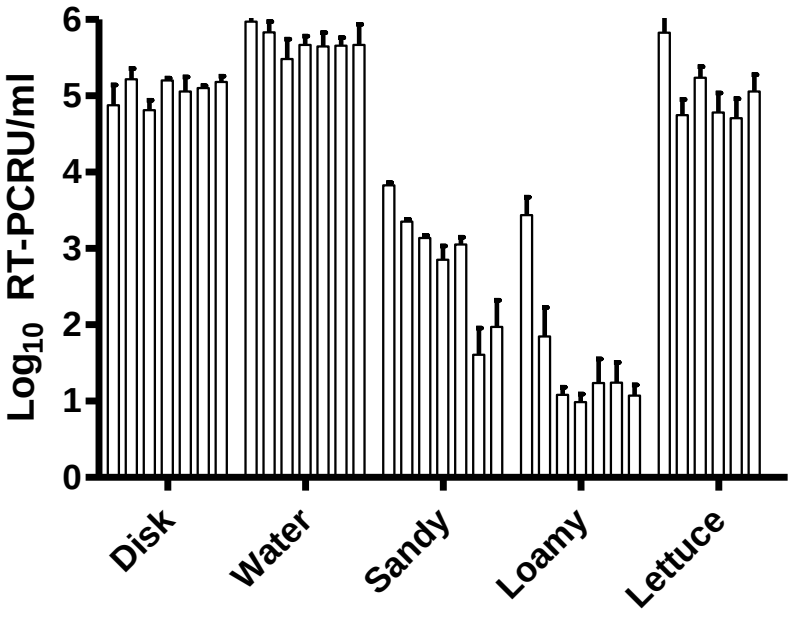

Log10 RT-PCRU/ml
0
1
2
3
4
5
6
Disk
Water
Sandy
Loamy
Lettuce

Figure 11. Persistence of MNV RNA on disk, lettuce, soil and in water

The reduction in genomic copy concentrations was determined by real-time RT-PCR. Time points for disk, water, soil (sandy, loamy) from day 0 to day 42 are as in Figures 7, 9, 10. Time points for lettuce for days 0 to 15 are as in Figure 8. The data were plotted using PRISM 4 software.

3.2 Stage B- Fate of MNV on Romaine lettuce in a field

3.2.1 Inoculum density estimation for the field trial

Prior to the trial, an experiment was conducted by Dr. Delaquis in British Columbia to estimate the approximate quantities of pathogens entering the soil. Briefly, the growing greenhouse lettuce and its neighbouring soil were inoculated with 10^8 CFU/ml of a culture of non-toxigenic *E. coli* O157:H7, which was diluted in 100 ml of distilled water. Considering that each plant and the adjacent soil would receive 100 ml of the *E. coli* irrigation, the soil would receive 10^5 CFU/g of *E. coli* pathogens. This result established the basis to calculate the inoculum density for other pathogens in the cocktail including MNV.

3.2.2 Recovery of virus from field samples

To irrigate the romaine lettuce plot, 12 L of the irrigation cocktail was required containing 10^7 PFU/ml of MNV-1. A stock of virus was prepared and determined to be 1.3 x 10^9 PFU/ml. Ninety two millilitres of the stock was diluted to 10^7 PFU per 1 ml of the irrigation cocktail. According to the result obtained from the *E. coli* experiment, each gram of soil or lettuce was expected to be inoculated with 10^6 PFU of MNV. However, the quantities of the infectious population recovered from the 3 samples of soil in this study from day 0 are 7.6 x 10^2, 5.7 x 10^2 and 1.5 x 10^2 PFU/g. It is noteworthy to consider that the mean recovery of the infectious MNV was estimated at 2 x 10^3 PFU per 25 g of lettuce which indicates less than 1% recovery. Given the fact that no infectious virus was detected from day 7 in both soil and on lettuce during the screening process, it was impossible to determine the decline in the surviving MNV population throughout the trial.

The recovery of FCV as an internal control was successful in all samples of lettuce and soil. The FCV was added to the lettuce and soil prior to extraction, in order to quantify the efficiency of viral recovery. The mean percent recovery of FCV ranged between 2-10% in all lettuce and soil samples.

3.2.3 Persistence of infectious virus and viral RNA on Romaine lettuce and soil in a natural farm environment

MNV spread and persistence were determined on lettuce and in soil under natural environmental conditions.

As described in the Materials and Methods, 25 g of each lettuce sample was examined for the surviving population of MNV every 7 days, firstly through conventional RT-PCR and secondly through sequencing and plaque assays. Table 5 shows that infectious MNV was detected in 14 samples (93%) recovered of lettuce on the day of inoculation. The samples from day 7 contained 6 conventional RT-PCR positives of which 3 (20%) were confirmed to be MNV by DNA sequencing, but none were detected by plaque assay. The samples from day 14 contained 3 conventional RT-PCR positives of which 2 (13%) were confirmed to be MNV but could not be determined to be infectious by plaque assay.

The soil specimens from the farm were extracted as for the laboratory studies. One gram of each soil sample was tested using conventional RT-PCR and the positives were plaque assayed and sequenced. Table 6 shows that infectious MNV was only found in samples from the day of inoculation. However, MNV nucleic acid was detected over the course of the experiment in 1 of 3 (33%) samples from days 7, 21 and 28, and in 2 (66%) of 3 samples from day 14.

	RT-PCR +	Sequencing +	Sequencing -	Infectivity +
Day 0	14/15*	14	0	14
Day 7	6/15*	3	3	0
Day 14	3/15*	2	1	0

** Total number of replicates tested for detection of MNV*

Table 5. MNV persistence on growing lettuce in a field of Romaine lettuce

The lettuce plot was inoculated with the irrigation cocktail using a watering can. MNV was detected on samples of lettuce leaves from growing lettuce by conventional RT-PCR and its infectivity was determined by MNV plaque assay.

	RT-PCR +	Sequencing +	Sequencing -	Infectivity +
Day 0	3/3*	3	0	3
Day 7	3/3*	3	0	0
Day 14	3/3*	3	0	0
Day 21	3/3*	3	0	0
Day 28	3/3*	3	0	0

** Total number of replicates tested for detection of MNV*

Table 6. MNV persistence in soil in a field of Romaine lettuce

The samples of soil were taken from within the lettuce rows. MNV was detected by conventional RT-PCR and its infectivity was determined by MNV plaque assay.

4 Discussion

To my knowledge, this is the first time that the persistence of NoV was evaluated on a farm and under natural conditions. The stability studies were initially planned to be carried out in one Eastern Canadian province and one Western Canadian province. This would have allowed me to monitor the spread and persistence of an infectious NoV on romaine lettuce growing in 2 types of soil differing in many characteristics including moisture and organic content. As described previously, an inoculum was prepared for both farms. Unfortunately, the sample plot on the farm in British Columbia was eaten by bears one week prior to the inoculation procedure. As reported here, samples from Nova Scotia were analyzed for this thesis and the British Columbia project was postponed.

4.1 MNV and susceptible cell lines

MNV can replicate *in vitro* in cell lines of hematopoietic lineages. A mouse leukemic macrophage cell line, RAW 264.7, has been widely used in enumeration techniques and propagation of MNV (130). I first employed RAW 264.7 cells in infectivity assays and for virus production. The preliminary data generated using these cells and previous studies in our laboratory raised concerns. RAW 264.7 cells are difficult to work with and occasionally stop growing and die when they have reached less than 50% confluency. This makes it impossible to perform a plaque assay. In addition, they have the tendency to cluster rather than forming a monolayer. I also observed that a rapid increase in acidity of the growth medium, possibly caused by high concentrations of cellular secretions, influences the lifespan of these cells. Finally, the small size of RAW 264.7 cells necessitates more cells to

form a monolayer. Moreover, the plaques formed using these cells are not easily visible in a microscope which makes it difficult to accurately enumerate the plaques.

In a publication by Cox *et al* in November 2009 (21), the susceptibility of 4 different cell lines of hematopoietic lineage including RAW 264.7 and BV-2 to MNV infection was investigated. They demonstrated that BV-2 and RAW264.7 had similar susceptibilities to MNV infection. Therefore I repeated previous experiments using the BV-2 cell line. The results and observations of my study are consistent with Cox et al (21). Unlike the RAW 264.7 cell line, BV-2 cells are easy to split, grow and occupy the available surface more quickly, and allow for the production of large numbers of cells in less time. This was particularly beneficial for the production of a large and high-titred virus stock (for the farm inoculation), or frequent plaque assays (for stability experiments). As compared to RAW 264.7 cells, the larger size of BV-2 cells also allows for more visible plaques.

Overall, the BV-2 cell line was more robust during laboratory manipulation. The use of the BV-2 cell line in this study required less time for cell maintenance, and provides support for the use of this microglial cell line in future MNV investigations.

4.2 Evaluation of recovery procedures

In the laboratory experiments, more MNV was recovered from the surface of lettuce leaves than from surface of disks. This is in agreement with a previous study by Dr. Mattison and colleagues in 2007 (81), which indicated that in a 20 minute period, air drying may have a greater impact on virus on the surface of the disks than on lettuce. The percent recovery from water was the least efficient, which could be attributed to method efficiency. Virus was concentrated from the water using a multi-step adsorption-elution method (14, 80) which could explain the greater loss of viral particles as compared to the direct elution methods used for the other three matrices (24). It is also possible that viruses attach to the sides of the vessels used during incubation and recovery. Spot inoculation provides higher recovery compared to other methods which is consistent with studies on recovery of food-borne viruses from food matrices (33). Overall, sufficient MNV was recovered from all matrices which allowed an effective evaluation of viral persistence over time (33).

I decided to apply the same RNA extraction method used in our laboratory studies (stage A) for our field experiment to maintain consistency in the results. For instance, I processed small volumes of soil (1g) since processing large samples of soil requires specific facilities and equipment and may lead to the loss of the intact viruses or damage to the capsid. A study by one of our collaborators (J. Brassard, personal communication) showed that in infectivity assays, the soil extracts from large volumes were damaging to tissue culture cells. Although many studies used 1-2 g of soil or biosolids for virus survival investigations (85) , in a study done by Tierney *et al* (116) where the persistence of poliovirus 1 in soil was assayed, recovery from 100 g of soil was successful with mean

recovery of 82%. To improve the recovery efficiency, testing a number of one-gram specimens from every soil sample could be considered.

4.3 Evaluation of MNV infectivity in/on matrices

In previous studies, Cannon *et al*. (17) found that MNV-1 was inactivated after 5 days on metal disks at room temperature whereas in this study, inactivation occurred after 12 days. This difference could be due to the composition of the inoculum preparation, which may have contributed to the survival of MNV in this study. Our inoculum could have been stabilized by the addition of bovine mucin, bovine serum albumin (BSA) or phosphate buffer to simulate soil load. Differences in extraction efficiency could also play a role (17, 56). Finally, the discrepancy could be related to the different time points sampled.

MNV applied to stainless steel disks shows a biphasic inactivation (Figure 7) with a greater initial reduction followed by a slower reduction phase. This indicates that a population of MNV could be more resistant to air drying, and also MNV would persist over a longer period of time if the experiment was extended. The biphasic inactivation has been reported for enteric viruses in other studies (96, 120). We tried both biphasic and linear analysis of the data points and concluded that linear reduction simplified the presentation of data.

MNV retained infectivity for longer period of time than the persistence previously observed for FCV on lettuce at room temperature, where infectious virus could only be detected for 4 days post-inoculation (81). MNV was inactivated more rapidly on lettuce than on steel disks, suggesting a potential role for lettuce cells in viral inactivation. This could also be explained if the viruses attach to or are internalized in the lettuce tissue (37, 109,

128). In a recent study by Wei *et al.*, (2011), MNV internalization has been observed in lettuce cells during irrigation (128).

In sandy and loamy soils over 42 days, the averaged data points almost completely overlap from both soils. Sandy soil contained less moisture and less organic contents than loamy soil. This indicates that factors other than moisture and organic content could influence virus survival in the soil environment. Hurst *et al*., (1980), have suggested that temperature and degree of virus adsorption are the dominant factors contributing to virus survival in soil (51). Yeager *et al.*(1979) (131) have proposed that viral inactivation in air-dried soil could be attributed to evaporation, but I prevented moisture loss in this study by wrapping the sealed cryovials in parafilm and I observed liquid in the loamy soil samples up until day 42 .

Ultimately, MNV survived longer in water than on any of the other matrices. In contrast to the soil data, this supports a role for moisture in prolonged persistence of virus. Other studies evaluating the persistence of enteric viruses on foodstuff have also considered moisture an effective factor for virus survival (1, 84). Overall, moisture could have less impact in the more complex soil environment than on steel or in water.

4.4 Detection of norovirus by molecular methods

The results of laboratory experiments showed that the MNV genome is more stable than infectious virus on various matrices tested. The results from the farm yielded similar conclusions indicating that there is little or no correlation between the number of infectious particles and the corresponding viral genomes. A number of studies on other enteric viruses, including FCV and poliovirus, are consistent with these data (5, 35). Gassilloud *et al.*, (2003) (35) observed a rapid decrease of infectious poliovirus in water by 4 $\log_{10}$ after 19 days, but calculated that a similar reduction in viral genomes would take 75 years.

In this study, real-time RT-PCR and conventional RT-PCR were used to detect MNV RNA in laboratory experiments. One of the problems we encountered in this project was the suspected degradation of the MNV standard for real-time RT-PCR indicating the contamination of reagents. As previously described, my attempt was to generate a ssRNA standard instead of using plasmid DNA to ensure the copy-number concentration of the targeted genome and percent recovery of the virus of interest were not overestimated (79). However, during the first attempts, the standard curve did not perform as expected. The predicted results were achieved once the stock of ssRNA standard was purified using Microcon YM-30 columns (Millipore) and a new dilution series was prepared. These results indicate the possibility that some impurity was present in the standards and inhibited the real-time RT-PCR reaction. This problem was not encountered when working with FCV.

Only conventional RT-PCR was used to detect MNV RNA on farm samples. We had previously observed that the use of the real-time RT-PCR method can result in both false positives and false negatives when processing environmental samples. There was a report of a similar problem from a laboratory testing farm samples for the presence of *E. coli* (J.

Brassard, personal communication). This can be attributed to the sensitivity of the real-time PCR method and therefore, various genetic materials from food samples might be amplified by non-specific binding of PCR primers. There may also be some inhibitors in food and environmental samples that reduce the detection efficiency of real-time RT-PCR by interfering with or inhibiting the RNA-directed DNA polymerase (RDDP) and/or DNA-dependent DNA polymerase (DDDP) activity. This was not observed as frequently with conventional RT-PCR. This may be related to a reported high sensitivity of the Taq exonuclease activity, necessary for the real-time assay, as compared to the polymerase activity, necessary for both assays (49).

Overall, although PCR methods are rapid and sensitive, interpretation of the results and confirmation of positives is not always conclusive since detection of genomic materials does not predict virus infectivity. This raises concerns whether an RT-PCR positive should be considered an indicator of public health risk. Nevertheless, foods containing NoV genome may still indicate the presence of fecal contamination. Therefore, molecular techniques are valuable as a diagnostic tool for NoV until a cell culture system becomes available.

4.5 Controls for Method Validation

Non-inoculated samples were evaluated as part of the method validation process using the same detection methodology as for the contaminated samples. If plaques or nucleic acids were detected in the negative control, this would indicate the presence of pre-existing norovirus on lettuce and in soil or from an unknown source or it would suggest problems with the detection methods. This method prevented the possibility of reporting false-positive results. All controls were confirmed to be negative for the presence of MNV by both RT-PCR and plaque assay.

In general, FCV is used as internal/positive control for enteric viruses, as initially proposed by Health Canada. FCV shares many genetic similarities with NoV, is not ubiquitously found in the environment and its detection methods have been well-studied and documented (13, 126). A known input concentration of FCV was added to each sample of lettuce and soil prior to the RNA extraction step (see Methods). It is important to detect FCV in all samples which suggests that the chosen method is effective in detecting enteric viruses. In addition, the mean percent recovery can be determined and compared to the recoveries obtained from tested samples which reduces the likelihood of false-negative results. Moreover, it was previously determined that the addition of FCV does not interfere with the detection of NoVs (44). However, it remains to be further elucidated whether the addition of internal controls reduces the detection of low levels of NoV in naturally contaminated products.

4.6 Monitoring of environmental conditions in field Romaine lettuce

The soil moisture (m^3 water/m^3 soil), temperature, relative humidity (%) and solar radiation (W/m^2) were monitored and recorded hourly throughout the trial (Appendix 1). The temperature ranged between 13.7 C° to 25.97 C° from day 0 to day 7. The relative humidity was 90.39% in average and the farm received 4.4 mm of rain on day 3 which did not affect the first samplings, however on days 8 and 10 the farm received 33.9 mm and 32.3 mm rain, respectively. Even with the rainy situation and relatively high humidity, the MNV genome could still be detected. That the virus did not retain infectivity longer could be attributed to many variables. Apart from extraction and recovery methods, many particles could be washed away and moved to lower layers of soil, while the temperature fluctuations may have an impact on the virus infectivity. UV light can also contribute to virus inactivation. The presence of the genome could also reveal that the loss of infectivity is related to the virus capsid. Although the temperature and relative humidity similar in the laboratory, MNV did not survive longer than 12 days. The temperature from day 7 to day 14 ranged between 10.99 C° to 26.73 C°. The averaged relative humidity was 90.6%. It was no longer possible to sample lettuce past day 14 due to excessive wilting.

Taken together, according to data from laboratory and farm, I came to the conclusion that persistence of infectious MNV on lettuce is less than on other matrices tested but infectious virus is present long enough to be considered a source of contamination. Finally the fate of MNV on lettuce should be determined under more controlled experimental conditions.

5 Conclusion and future work

My laboratory studies examined the persistence of virus on a stainless steel surface which mimicked the potential contamination of farm equipment, food-contact surfaces or eating utensils. In addition, I examined the survival of infectious MNV in water and soil.

My findings in water, on vegetables and food-contact surfaces indicate that NoV has the potential to persist in the environment and pose a risk for food safety. The data presented here, in combination with findings of NoV genomes in bottled water (10, 11, 35) and outbreaks originating from contaminated drinking water (65, 95) raise concerns that water could readily transmit NoV infection. Disinfection treatments should be developed to inactivate infectious NoV in addition to other pathogens. Overall, my data provide valuable information to evaluate the risk for viral transmission from fresh produce and environmental matrices.

This study provides valuable information about NoV stability along the farm-to-fork continuum. The laboratory and farm studies of the persistence of MNV on smooth surfaces such as lettuce and metal versus more variable matrices such as soil and water provide evidence for modelling the potential public health risks from a contaminated agricultural environment.

Future work will consider the preliminary data that were generated in our farm study in order to model the spread of MNV in a lettuce field under more efficient and controlled farming or processing conditions. Also, future studies may consider having more frequent samplings with shorter intervals.

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