ABSTRACT

Human noroviruses are the leading cause of foodborne gastroenteritis in the United States. Recent research has focused on norovirus attachment to host-cell carbohydrates. This area of research increases understanding of the first step of norovirus replication in host cells; it also has been extended to explaining norovirus capsid structure and virus evolution, as well as norovirus attachment to foods and has been used in developing methods of detecting human noroviruses. This article provides a review of research conducted on human norovirus attachment, specifically on the attachment of noroviruses on foods, including original research addressing the role of spinach and green onions in disease caused by human norovirus and a common human norovirus surrogate, murine norovirus. The objective of this study is to assess the applicability of an ELISA attachment assay to predict infectivity of a genogroup II human norovirus and to determine if spinach and green onion homogenates interfere with the attachment of the virus to the carbohydrate coating. Attachment assay results for heat-treated human norovirus indicated decreasing attachment with increasing heat treatment, which likely correlates with loss of infectivity. Additionally, the presence of spinach and green onion homogenate did not affect attachment of human norovirus or murine norovirus to porcine gastric mucin.
INTRODUCTION

Human noroviruses cause 58% of gastroenteritis in the United States and are suspected to be the leading cause of foodborne illness (37). Noroviruses typically cause a self-limiting acute diarrheal disease lasting 2–3 days; however, more serious cases have been responsible for 26% of hospitalizations and 11% of deaths caused by foodborne illnesses (37). Although the ways in which noroviruses infect human gastrointestinal cells are not completely understood, in 2002, human noroviruses were discovered to bind to histo-blood group antigens on human gastrointestinal cells (32). This finding opened a door to research aimed at understanding the beginning stages of norovirus replication, structural differences and evolutionary strategies and applying this understanding to development of more precise detection methods as well as evaluation of human norovirus attachment to foods.

Norovirus attachment to histo-blood group antigens

The initial steps of virus infection involve attachment of the virus to host cellular receptors, followed by entry of the virus through the cell membrane and subsequent release of the genetic material into the cytoplasm. Carbohydrate moieties of host cell glycoproteins, glycolipids such as sialic acid, and proteoglycans comprise a widely used strategy of virus attachment to epithelial cells (5). The attachment of virus to host cellular receptors involves recognition of the specific cellular receptor. Histo-blood group antigens (HBGA) are receptors on human gastrointestinal cells to which NoV binds (19, 20, 32). Often, viral penetration is mediated by a membrane protein that interacts with the virus specifically as a receptor or co-receptor, for example, the norovirus surrogate feline calicivirus binds to sialic acid on host cells (39) and to a cellular membrane protein, junctional adhesion molecular 1 (JAM1) (31, 34). With human noroviruses, a 105-kDa membrane protein, designated as NV attachment (NORVA) protein, present in mammalian cell lines including Caco-2 cells was shown to interact with human norovirus-like particles (40). The function of this protein–protein interaction between NORVA and NoVLPs has yet to be defined (43) and attempts to extract and purify the protein have been unsuccessful (41).

Knowledge of HBGA binding patterns of human noroviruses have been elucidated through studies using in vitro binding and blocking assays with recombinant-like particles, human volunteer challenge studies, and outbreak investigations (43). Histo-blood group antigens, a major group of carbohydrates that are determinants of human and animal blood types, are abundant in bodily fluids including blood, saliva and milk. HBGAs, found on cellular surfaces and mucosal epithelium, are complex carbohydrates present on the outermost ends of N- or O-linked glycans or glycolipids (43). Different norovirus strains have varying receptor-binding profiles associated with the ABO, secretor, and Lewis HBGA types as determined by human volunteer challenge studies (43). In vitro binding assays revealed that most GI1.4 strains recognized saliva of all ABO secretors, which represent approximately 80% of the world’s population, indicating an important reason for the predominance of this genotype over those that have a narrower target population (43). Of all norovirus genotypes tested, GI1.4 was observed to bind to the widest variety of HBGAs (19).

In addition to HBGAs, human noroviruses have been shown to bind to porcine gastric mucin (PGM) (48) and heparan sulfates (41). Porcine gastric mucin (PGM) contains type A, type O and type Lewis b HBGA, to which NoV has been shown to bind (48, 49). PGM also contains protein (20%) and carbohydrates such as hexosamine (37%), hexoses (27%), fucose (10%) and sialic acid (6%) (14). Studies using PGM to assess the mechanism of norovirus attachment showed that the binding of NoVLP to PGM was completely inhibited after periodate oxidation of carbohydrates at all concentrations, while boiling the proteins had no effect (14). This provided further confirmation that NoV attachment to PGM occurs through the carbohydrate moieties and not through protein–protein interactions (49).

Norovirus capsid structure and interactions with antibodies and receptors

Following the studies linking noroviruses to various cell surface receptors, X-ray crystallography, cryoelectron microscopy, NMR and genetic mutagenesis techniques were used to advance knowledge of the crystal structures and binding interfaces of noroviruses with host cellular receptors (4, 6, 10, 11, 42, 45, 48). The major structural protein of noroviruses, VP1, is composed of two domains, the shell (S) domain, which forms the core of the icosahedral shell (3), and the protruding (P) domain, which forms arches extending from the shell. The P domain, located at the most exterior surface of the viral particle, is responsible for host interactions and the immune response (6). The P domain is composed to two subdomains, P1 and P2, which correspond to the leg and head of the arch-like P dimer (35). Binding has been observed to occur at the outer pocket of the P domain protrusions (6, 42). This HBGA-binding pocket includes a group of scattered amino acids that interacts with the oligosaccharide residues of the HBGA receptor (6, 10, 11).

Through comparing the structures of the P domain bound to A tri saccharides, evidence of different receptor-binding motifs were observed for both GI and GII noroviruses (6). Bu et al. (6) determined that both GI and GII noroviruses bound to the same A and H antigens and the binding interfaces were located on the same region of the P2 domain, but the amino acids involved were completely different. Sequence alignment of noroviruses showed that key residues for HBGA binding are highly conserved among strains within the genogroups, but not between two genogroups; the remaining sequences of the P2 subdomain are highly variable (46). This suggests that interactions with HBGAs play an important role in the evolution of noroviruses (44).

Norovirus attachment in foods

Knowledge gained from attachment research has also focused on the possible attachment of human noroviruses to foods. Because noroviruses are transmitted through the fecal-oral route, it would be of evolutionary advantage for the viruses to bind easily to a broad range of vehicles, including foods, so as to be spread more efficiently (26). Additionally, the attachment of noroviruses to foods likely promotes persistence and survival. The study of viral attachment to foods has presently focused on two groups of foods: shellfish, particularly oysters, and produce, particularly romaine lettuce. Both of these groups are commonly implicated in norovirus outbreaks worldwide (1, 2, 7, 15, 29, 33, 57, 62). Understanding virus attachment mechanisms is key to developing effective detection methods as well as optimizing intervention strategies.
Early efforts to understand the mechanism of virus attachment to lettuce focused on electrostatic forces, in particular the role of the isoelectric point of the virus (58, 59). Virus attachment to lettuce was first assessed by Vega et al. (59) using bacteriophages MS2 and ΦX174, feline calicivirus, and echovirus 11 at various pH levels. The authors hypothesized that the attachment of these viruses to lettuce would depend on the isoelectric point of the virus, because the ultimate charge of a virus would depend on the amino acid residues on the virus surface and on the pH of the surrounding medium. The four viruses had varying attachment patterns to lettuce despite having similar isoelectric points, which indicated that the isoelectric point of the virus was not the factor governing their attachment to lettuce. The authors then compared viral attachment to lettuce with Tween 80 added and NaCl added, to test for hydrophobic interactions and electrostatic interactions, respectively (58). Vega et al. (58) concluded that electrostatic forces are the primary force involved in the adsorption to lettuce.

Since the discovery of norovirus attachment to histo-blood group antigens, research on the attachment of enteric viruses on foods has shifted from non-specific to specific interactions. Early on, the attachment of poliovirus to oysters was shown to involve binding of the virus particles to carbohydrates moieties (13). Similarly, GI NoV were also found to bind to carbohydrates in oysters, specifically histo-blood group antigens present in the gastrointestinal cells of oysters (24, 48, 51), as observed in ELISA attachment assays and confocal microscopy. Oyster stomach and digestive tissues were reported to contain type A-like HBGAs to which NoVs bind (24, 48). Type A-like HBGAs have been reported to bind to multiple NoV strains (21), suggesting that other strains of NoVs bind to oysters. In competitive binding assays, human saliva type A HBGA and anti-type A HBGA MAb inhibited the binding of NoVLPs to the oyster stomach and digestive tissues. Specifically in oysters, enteric viruses, including MNV and HAIV, have been observed to bind to phagocytic blood cells (hemocytes) (36). These results suggest that NoV binding to oyster tissue is mediated by specific ligand-receptor interactions and not just physical entrapment. Through this HBGA binding, it is hypothesized, oysters bio-accumulate NoVs during the course of filter feeding (24, 51). Mutant human norovirus virus-like particles (NoVLPs) with an alanine substitution in the P2 domain of VP1 prevented the binding to oyster tissue (24), suggesting similarities to the mechanisms of recognition of human tissues by NoVs.

Tian et al. (51) assessed NoV binding to HBGAs in a variety of bivalve filter feeders, including a range of oyster species, clams and mussels. Through ELISA binding assays using MAbs specific for different HBGAs, it was revealed that both type A-like and type O-like HBGAs were present in all oyster species and in Manila clams; however, blue mussels contained only type A-like HBGAs. Although both type A-like and type O-like HBGAs are present, competitive assays indicated that type A-like HBGAs appear to have a dominant role in the NoVLP binding in oysters (51). The genetic diversity of NoVs, highlighted by differences between GI and GII binding to human HBGAs, is similarly observed in the binding capacity to HBGA structures within oysters. GI NoVs bind to type A-like HBGAs in oyster digestive tissues, whereas GII NoVs, in addition to binding to digestive tissue by a type A-like HBGA, are also able to bind to gills and mantle tissue sections by a sialic acid α2,3 linkage (30).

The role of seasonality and glycogen levels in NoV attachment to oysters was hypothesized, as oyster glycogen is present in high concentrations from November to March, which coincides with the time of seasonal outbreaks of norovirus infections (24, 52). In assessing the role glycogen plays in norovirus binding, it was observed that norovirus attachment to oysters does not have a seasonal pattern (52). Contrary results were observed by Maalouf et al. (30); GI VLPs were able to bind more much efficiently to oysters during the first five months of the year than during the rest of the year, and GI strains are most regularly involved in oyster-related outbreaks from January to May.

Wei et al. (61) observed, by use of confocal microscopy, that SYBR gold labeled MNV attached to Romaine lettuce surfaces. Virus was pipetted directly onto lettuce or the lettuce was agitated in virus suspension, and the virus was found in stomata, along the cut edges and on leaf surfaces; biosolids were shown to promote this attachment of MNV to lettuce (61). Porcine sapovirus, as a surrogate for human noroviruses, were also found to attach to Romaine lettuce and remain infectious to lettuce one week after storage at refrigeration temperatures (60). Confocal microscopy has also shown that NoVLPs localize in clusters along the veins rather than being equally distributed through the leaf (16). The specific attachment of NoVLPs to Romaine lettuce extract was further elucidated through ELISA methods (16). Extracts of Romaine lettuce leaves coated to ELISA plates were observed to bind the NoVLPs in a dose-dependent manner, but Romaine lettuce extract did not bind the norovirus-like particles by HBGA. Additionally, Romaine lettuce extract was not competitive with NoVLP binding to PGM, suggesting that non-HBGA molecules in Romaine lettuce bind to NoVLPs via binding sites different from the defined binding pocket of the virus particle. Proteins were also suggested to have some role in the binding to NoVLP to Romaine lettuce exudate, as binding was increased slightly by oxidation but decreased slightly by boiling.

To further understand the attachment of human noroviruses to Romaine lettuce, Esseili et al. (14) assessed the binding of NoVLPs to cell wall material carbohydrates of Romaine lettuce leaves. Through studies of oxidation of carbohydrates and boiling of proteins, it was determined that NoVLP attachment in young leaves was primarily associated with proteins whereas in older leaves it was primarily through carbohydrates (14). This difference in the binding mechanisms of NoVLPs to cell wall material could be the result of different sugar abundances or differences in composition between young and old leaves. This is supported by previous studies showing that the plant cell wall changes continuously in different developmental stages (8). NoV-lettuce interaction is mediated by various carbohydrate moieties present in the plant cell walls (14). Minor binding to cell wall proteins was also observed (14), which is supported by the observed binding of NoV G1.1 binding to unknown proteinaceous components released from the surface of lettuce plants (16).

Esseili et al. (14) investigated the specific inhibition of carbohydrate moieties, using specific monoclonal antibodies against carbohydrates of human HBGAs. The antibody against the A antigen was the only antibody that significantly inhibited VLP binding to PGM, and none of the MAbs inhibited binding of VLPs to lettuce leaves. This further confirms the hypothesis of Gandhi et al. (16) that NoVLPs bind to Romaine lettuce leaves differently from the binding to HBGAs and PGM. In addition to assessing norovirus attachment to Romaine
lettuce; extracts prepared from cilantro, iceberg lettuce, celery, spinach, green onions, clover sprouts, and raspberries were used to coat ELISA wells to measure NoVLP binding; however, none of these bound NoVLPs as well as Romaine lettuce (16). In a study assessing the ability of washing to remove human noroviruses from different types of produce, it was observed that a greater percentage of GI NoV was removed from raspberries (95%) than from lettuce (75%), suggesting stronger binding of NoV to lettuce (55). The amount of virus removed by washing significantly decreased when produce was washed with acidic electrolyzed water (AEW, pH 2.2–2.4), suggesting enhanced binding of norovirus to raspberries and lettuce due to ionic interactions (55).

Using attachment to detect norovirus detection and to assess infectivity

The attachment properties of noroviruses have been exploited for use in detection assays (9, 17, 50, 53, 54) in which HBGAs or PGM were conjugated to magnetic beads that were then used to isolate noroviruses from water, sewage/wastewater and lettuce. Advantages of isolating noroviruses through HBG attachment methods include both ability to detect intact capsids and increased sensitivity. Recently, a study used viral binding properties in tandem with RT-PCR to discriminate between infectious and non-infectious noroviruses in a binding-based RT-PCR assay (26). Binding-based RT-PCR was investigated for its ability to distinguish between infectious and non-infectious virus particles, with the hypothesis that binding to a receptor followed by RT-PCR would better indicate viral infectivity, eliminating the detection of viruses with damaged capsid or RNA. In this study, attachment of both infectious and inactivated (by H2O2 or heat) MNV and NoV was assessed using ganglioside GD1a, the attachment receptor of MNV on RAW cells, and PGM or Caco-2 cells, respectively. MNV results were confirmed by plaque assay. For the inactivation of MNV by heat (70°C, 3 min) and H2O2 (2.1%, 5 min), the plaque assay showed reductions of > 6 log PFU/mL. Reductions of 1 log were obtained by RT-PCR after heat inactivation as well as by cell binding RT-PCR.

The RT-PCR assay was shown to be a better indicator of infectivity for heat-treated MNV and human norovirus GII.4 (70°C, 2 min) compared with RNase treatment of virus followed by RT-PCR (28). The RNase RT-PCR methods measure the ability of the virus capsid to protect the interior viral RNA genome from digestion by RNase (56), while the cell-binding RT-PCR assay is based on the ability of the virus capsid to bind to specific receptors. This binding is essential for viral infection (26), likely because the structure of the protruding (P) domain on the capsid is denatured by heat before the occurrence of any severe damage on the capsid that would enable the entry of RNase One and subsequently RNA degradation (28). Using virus attachment to PGM coated beads coupled with viral detection by qRT-PCR, Dancho et al. (12) investigated whether human norovirus G1 and GII.4 binding ability after treatment with heat, high-pressure, and UV was correlated to infectivity. Results showed that these virus treatments affected capsid integrity enough to interfere with attachment to PGM (12); however, human clinical trials for UV and heat-treated noroviruses have not been conducted, and it cannot be said with certainty that altered binding properties definitely resulted in loss of norovirus infectivity. Although none of these assays identifies a reduction in virus titer comparable to results obtained with infectivity assays, they represent an improvement in evaluation of norovirus infectivity compared with results obtained with standard RT-PCR and they enhance our understanding of the steps involved in virus infectivity.

Recently, we published research using murine norovirus (MNV) as a surrogate for human norovirus, in which an ELISA method was utilized to assess attachment through binding to host cell receptors, and MNV attachment was correlated to infectivity as determined by a plaque assay (18). ELISA plates were coated with porcine gastric mucin and untreated, heat-, high pressure-, ozone- or UV-treated MNV was added, followed by monoclonal anti-MNV IgG antibody. Through use of this ELISA attachment assay on heat-treated murine norovirus (MNV), a positive correlation was observed between virus attachment patterns and inactivation of virus. While this correlation was not observed for other processing methods, including high pressure-, ozone- and UV-treated MNV, this assay has the potential to be useful for predicting human norovirus infectivity after heat treatment. The objective of the short study described here is to assess the applicability of the ELISA attachment assay to a GII NoV and to assess whether food matrices interfere with attachment of the virus to the carbonate coating on the ELISA plate. In particular, attachment of MNV and NoV suspended in spinach and green onions was assessed.

MATERIALS AND METHODS

Human norovirus

GII NoV were isolated from fecal samples (generously provided by Megan Davis, South Carolina Department of Health and Environmental Control). NoV was extracted from stool samples by a protocol similar to Straub et al. (38). Stool samples were suspended in PBS (0.01 M) to obtain a 10–20% stool suspension. The suspension was vortexed for 60s, centrifuged at 1000 x g and processed through at 0.22 micron-filter to remove bacterial contamination. NoV was purified by a polyethylene glycol (PEG) precipitation as described by Lewis and Metcalf (25). Briefly, viral suspensions were added to PEG 8000 (Fisher Scientific, Waltham, MA) to obtain a final concentration of 8% (wt/vol), stirred for 2 h at 4°C, and centrifuged at 10,000 x g for 20 min. The PEG supernatant was discarded and the viral pellet resuspended in 0.15 M Na2HP04 (pH 9.0) and then shaken for 20 min at 250 rpm before centrifugation at 10,000 x g for 30 min. The pH of the supernatant was adjusted to 7.4 and stored at -80°C until used. NoV was heat treated at 20, 50, 60, 70, 80 and 100°C for 5 min in 200 µL aliquots of the supernatant in 0.2 mL PCR tubes in an Eppendorf Mastercycler (Eppendorf, Duesseldorf, Germany).

Attachment – enzyme-linked immunosorbent assay (ELISA)

ELISA plates (CoStar, Corning, NY) were pre-coated with a 10% solution of poly-L-lysine (Sigma Aldrich, St. Louis, MO) in Tris buffered saline (TBS, 100 mM, pH 7.4). After a 30-min incubation, ELISA plates were washed three times with sterile ddH2O, and coated with 100 µL of 10 µM/mL PGM (Sigma Aldrich, St. Louis, MO) in TBS and incubated for 24–48 h at 4°C. Unbound PGM was removed by washing three times with ddH2O and the wells were then blocked for 2 h at room temperature with Animal-Free Blocking Buffer (Vector Labs, Burlingame, CA). After removal of the blocking buffer, wells were
FIGURE 1. Attachment of heat-treated GII human norovirus to porcine gastric mucin coated ELISA plates. Positive attachment is indicated by a P/N ratio ≥ 2, shown by a bold line, confirming that treated virus can be recognized by monoclonal antibodies. P/N Ratios are not statistically different (P > 0.05).

TABLE 1. RNase treatment of heat-treated GII human norovirus to determine capsid integrity followed by RT-PCR. Nucleic acid presence is expressed as the number of intact capsid samples/destroyed capsid (n=2)

<table>
<thead>
<tr>
<th>Temperature (°C)</th>
<th>20°C</th>
<th>50°C</th>
<th>60°C</th>
<th>70°C</th>
<th>80°C</th>
<th>100°C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Attachment (P/N Ratio)</td>
<td>2/0</td>
<td>2/0</td>
<td>2/0</td>
<td>2/0</td>
<td>0/2</td>
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FIGURE 1. Attachment of heat-treated GII human norovirus to porcine gastric mucin coated ELISA plates. Positive attachment is indicated by a P/N ratio ≥ 2, shown by a bold line, confirming that treated virus can be recognized by monoclonal antibodies. P/N Ratios are not statistically different (P > 0.05).
washed three times with ddH2O. MNV or GII Human norovirus samples
diluted in TBS (10-1 dilution) were added to wells and incubated at
37°C for 1 h. Unbound virus was removed and wells were washed three
times with ddH2O before the addition of 1:1000 dilution of monoclonal
mouse anti-MNV IgG (generously provided by Dr. Christine Wobus,
University of Michigan) or anti-NoV mouse IgG P408 monoclonal
antibody (Kim Laboratories, Rantoul, IL) diluted in TBS. Anti-MNV IgG
or anti-NoV IgG was allowed to incubate at 37°C for 1 h before unbound
anti-MNV IgG was removed by washing the wells with TTBS (TBS with
0.5% Tween-20). Anti-MNV or anti-NoV antibodies were detected by
horseradish peroxidase conjugated goat anti-mouse IgG secondary
antibody (Promega, Madison, WI) diluted 1:2500 in TTBS. The secondary
antibody was incubated at 37°C for 1 h. Unbound secondary antibody
was removed by washing three times with TTBS, and bound HRP-
conjugated goat anti-mouse antibodies were detected by adding 80 µL
of 3,3′,5,5′-tetramethylbenzidine (TMB) liquid substrate (Promega).
Samples were incubated at room temperature for 5 min before the
reaction was stopped by addition of 1N HCl, and absorbance was read
at 405 nm on a plate reader.

RNase treatment of heat-treated NoV GII

To determine whether viral capsids were intact after treatment,
heat-treated NoV GII was subjected to RNase prior to RT-PCR, as
previously described (18). Briefly, NoV GII was subjected to RNase (20
units) for 30 min at 37°C, after which RNase inhibitor (20 units) was
added. Viral RNA was released by treatment at 99°C for 5 min. RNase
inhibitor was added again and one-step RT-PCR (Qiagen, Valencia, CA)
was performed on serially diluted virus in 25 µL volumes containing
5 µL 5 Buffer, 1 µL dNTP (10mM), 2 µL RNA, 1 µL enzyme mix, 0.2 µL
RNase Inhibitor (Fisher Scientific, Waltham, MA) and 9.8 µL RNase-
free H2O. Primers for NoV GII were designed by Primer3 and target the
VP1 region (VP1-FP3 – 5′-TGGGTGCTCCAAGGTTACC-3′ and VP1-RP3-
5′-CTGGACTGCTCCTTGGTAG-3′) to produce a product of 196-bp.

Effect of spinach and green onions on viral attachment to PGM

To assess MNV and NoV attachment to spinach and green onion
plants, one mature plant was homogenized in TBS (10 mL), coated on
ELISA plates and incubated for 24–48 h at 4°C. The attachment ELISA
assay was performed as described previously (18). To assess the effect
of plant homogenate on results of the MNV and NoV attachment assay,
ELISA plates were pre-coated with poly-L-lysine and coated with PGM
and then incubated for 24–48 h at 4°C as described previously. MNV
and NoV suspended in spinach and green onions homogenized in TBS
were added to wells after blocking and washing. Attached MNV and NoV
were detected by anti-MNV and anti-NoV IgG and HRP-conjugated goat
anti-mouse secondary antibody as described previously.

Data analysis

Virus attachment is represented as the positive to negative ratio
(P/N), in which the OD405 readings of sample wells were compared
to those of the negative control wells. Negative control wells included
wells without PGM, spinach or green onion homogenate, or wells without
the primary antibody. A P/N Ratio ≥ 2 was considered to be positive
attachment (18, 48). Three technical replicates were performed for
three biological replicates and the results recorded as the mean and
standard deviation of the mean. Statistical analysis included the
difference of means t-test on Microsoft Excel 2010. Attachment was
considered significant if P ≤ 0.05.

RESULTS AND DISCUSSION

Attachment of heat-treated human norovirus GII

Human norovirus genogroup II attached positively (P/N Ratio > 2)
to porcine gastric mucin after heat treatment (Fig. 1), P/N ratios were
2.70 ± 0.83, 2.30 ± 0.39, 2.26 ± 0.77, 2.01 ± 0.75, 2.09 ± 0.44, and
2.04 ± 0.29 for 20, 50, 60, 70, 80, and 100°C, respectively. However,
attachment P/N ratios were close to the cut-off value of 2 at 70, 80 and
100°C. Results with RNase treatment (Table 1) indicate the NoV capsid
does not remain intact at 80 and 100°C; norovirus RNA is not detected
at these temperatures, since the viral capsid is no longer intact,
which allows RNase degradation of the viral genome. This pattern of
attachment with heat-treated human norovirus is similar to that
observed for heat-treated MNV (18), which may indicate that NoV is no
longer infectious when the P/N ratio of attachment to porcine gastric
mucin is less than or equal to 2. However, this is difficult to predict, as
to date human clinical trials assessing NoV infectivity have not been
performed on heat-treated NoVs.

Effect of spinach and green onion homogenate on MNV and NoV
attachment to porcine gastric mucin

The effects of food matrices on results of the attachment assay
were assessed using spinach and green onion plants for MNV (Fig. 2)
and NoV (Fig. 3). MNV attached strongly to spinach and green onion
when the plant homogenate was coated on the ELISA plates, with
P/N values of 13.60 ± 0.24 and 14.50 ± 0.33, respectively. Despite
the significantly greater (P ≤ 0.05) attachment of MNV to both plant
homogenates, MNV suspended in spinach and green onion homogenate
was able to positively attach to porcine gastric mucin, where P/N ratios
were 2.75 ± 0.13 and 3.05 ± 0.29 (Fig. 2). Similarly, NoV was shown to
positively attach to spinach and green onions when plant homogenate
was coated to ELISA plates, with P/N values of 3.82 ± 0.27 and 3.73
± 0.46, respectively (Fig. 3). While there is a positive attachment
(P/N ≥ 2), this interaction of NoV with the spinach and green onion
homogenates, as indicated by the P/N Ratio, is significantly less
(P ≤ 0.05) than it was for MNV, possibly because of the difference in
the binding/attachment properties and structure of the two viruses
(22, 23, 43, 47). When NoV was suspended in spinach and green onion
homogenate, NoV positively attached to PGM, with P/N ratios of 3.64 ±
0.76 and 2.56 ± 0.52, respectively.

These results indicate that, despite the attachment of these
viruses to produce, this attachment does not interfere with attachment
to PGM, suggesting that this assay could be used for assessing
infectivity of heat-treated virus or of virus in some food commodities.
It is important to assess any inhibition by compounds in plant material
that could interfere with the assay. Attachment of MNV and NoV to
extracts of spinach was demonstrated by Gandhi et al. (16), who
showed that recombinant human norovirus-like particles (rNoVLPs)
attracted positively to spinach; however, the attachment of rNoVLPs to
spinach was weaker than the attachment of rNoVLPs to Romaine lettuce
FIGURE 2. Attachment of murine norovirus to spinach or green onion homogenate (□) or attachment of murine norovirus suspended in spinach or green onion homogenate to porcine gastric mucin (■). Positive attachment is indicated by a P/N ratio ≥ 2, shown by a bold line, confirming that murine norovirus virus can be recognized by monoclonal antibodies.

FIGURE 3. Attachment of GII human norovirus to spinach or green onion homogenate (□) or attachment of NoV suspended in spinach or green onion homogenate to porcine gastric mucin (■). Positive attachment is indicated by a P/N ratio ≥ 2, shown by a bold line, confirming that GII human norovirus can be recognized by monoclonal antibodies.
extracts. Additionally, Gandhi et al. (16) did not observe attachment of rNoVLPs to green onions. Food items such as oysters and Romaine lettuce, which have been shown to bind to NoVs through carbohydrates similarly to HBGA attachment of NoVs (14, 24, 49), may affect attachment to PGM and pose a challenge to predicting infectivity using this attachment ELISA. Li et al. (27) tested the binding ability of GII.4 NoV P particles on various types of shellfish, vegetables, and fruit juices in a saliva-binding enzyme-linked immunosorbant assay (ELISA). As predicted, oysters had negative effects on NoV P particle attachment to human saliva, suggesting that the shellfish extracts blocked the binding sites of the NoV P particles, thus reducing the HBGA-binding levels. No inhibition of binding effects were observed for the fresh produce, including strawberry, raspberry, blackberry, blueberry, cherry tomatoes, spinach, and romaine lettuce (27). It should be taken into consideration that the behavior of NoV P particles may not be indicative of human norovirus particles. The components of food such as carbohydrates will vary between commodities and should be assessed for each intended use.

CONCLUSION

The studies described here represent a broad view of the challenges to understanding how NoV interacts with foods. This knowledge can help lead to the development of more effective barriers against initial contamination and limit spread or transmission of NoV among food products. Attachment of NoV is complex, as shown by the various carbohydrates and proteins that may be involved. These challenges are greatly increased by the inability to culture human NoV and the reliance on surrogates, which, although they may provide a better model for inactivation, are weaker models for attachment.

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