Variability of Virus Attachment Patterns to Butterhead Lettuce

EVERARDO VEGA,1 JEANON SMITH,1 JAY GARLAND,2 ANABELLE MATOS,3 AND SURESH D. PILLAI1*

1Food Safety and Environmental Microbiology Program, Poultry Science Department and Institute of Food Science & Engineering, Texas A&M University, College Station, Texas 77843; 2Dynamac Corporation, Kennedy Space Center, Florida; and 3U.S. Department of Agriculture Research Service, Eastern Regional Research Center, Wyndmoor, Pennsylvania, USA

ABSTRACT

Enteric viruses account for most foodborne illness in the United States. The objective of this study was to determine whether the isoelectric point (pI) of viruses such as feline calicivirus (FCV), echovirus 11, and bacteriophages ϕX174 and MS2 had any effect on their attachment to butterhead lettuce. The adsorption of virus particles to the lettuce was variable. Bacteriophage MS2 was the only virus that fit the current Derjaguin-Landau-Verway-Overbeek model of virus attachment. Echovirus 11 had the highest affinity to lettuce surface. Echovirus 11 appeared to exhibit reversible attachment above its pI, whereas below its pI strong adsorption was observed. Adsorption of FCV was at its maximum above its pI. Bacteriophage ϕX174 exhibited the most complex adsorption pattern, with attachment occurring only at the pH extremes (pH 3.0 and 8.0). These results suggest the current model for virus adsorption to sediment does not adequately explain the attachment of virus to lettuce. Importantly, the results indirectly suggest that current sample processing methods to recover viruses from lettuce may differentially select for the recovery of only certain virus types.

The Centers for Disease Control and Prevention has recognized that among the foodborne disease outbreaks between 1993 and 1997, more than half of the cases of “unknown etiology” exhibited characteristics of viral illnesses (17). In 2000, viral agents accounted for 28% of all documented foodborne illness cases, whereas bacterial agents accounted for 25% of all foodborne disease outbreaks (5). Mead et al. (16) suggested that enteric viruses may actually account for as much as 67% of all foodborne disease–related gastroenteritis in the United States. The large number of enteric virus infections can be attributed in part to their low infectious doses (≤100 PFU), their stability in the environment, and our relatively limited knowledge about their presence in foods (2, 3, 23).

Although the incidence of foodborne illnesses associated with fresh produce is relatively low, the number of outbreaks associated with fresh produce has recently doubled (4). We recently reported that carrots, cilantro, and parsley can harbor fecal indicator viruses (9, 10). Lettuce is of particular concern among salad crops, because it is consumed in relatively large quantities with minimal preparation, has a large surface area (hence, greater pathogen attachment sites), and is grown in close proximity to the soil. Lettuce has been implicated in a large viral disease outbreak. In 1988, a hepatitis A outbreak that involved more than 200 people was attributed to lettuce being contaminated via irrigation water (20).

The initial attachment of enteric viruses to salad crops and herbs is a key step in the contamination chain of events. Understanding the factors (physical, chemical, and biological) that control the attachment process can provide insight into appropriate intervention methods that can be used to either prevent attachment or remove the attached viral pathogens. A significant amount of information is related to factors that control the attachment of enteric viruses to soil and aquifer sediments. Factors such as virus type, pH, ionic concentration, presence of multivalent cations, and organic matter are thought to be involved (6, 7, 11, 18, 21, 24). The factors that control enteric virus attachment to salad crops have, however, not been adequately studied. The attachment of enteric virus particles to lettuce leaf surfaces can be envisioned as involving both kinetic adsorption and equilibrium attachment processes (11). Since the isoelectric point (pI) of a virus can influence the net surface charge of a virus at a particular pH, we hypothesized that the pI of the virus is a controlling factor that dictates the attachment of enteric virus particles to butterhead lettuce surfaces. Because viruses consist of nucleic acid surrounded by a protein coat, a virus particle can be thought of as a protein with a defined surface charge. The ultimate charge of a virus would therefore depend on the amino acid residues on the virus surface and the pH of the surrounding medium (7, 12, 18, 24).

To delineate the factors that control the nonspecific attachment of enteric viruses to produce surfaces, the attachment of viruses to lettuce surfaces was studied using batch experiments. The primary objective of this study was to determine if the pI of the virus controlled their attachment to the lettuce surface. The viruses that were studied included model viruses (bacteriophages and feline calicivirus [FCV]) and a known enteric virus (echovirus 11). Only if the factors that control virus attachment to lettuce are identified can effective and scientifically based washing or virus recovery protocols be developed.

*Author for correspondence. Tel: 979-845-2994; Fax: 979-845-1921; E-mail: spillai@poultry.tamu.edu.
TABLE 1. List of viruses used in the study and their physicochemical characteristics

<table>
<thead>
<tr>
<th>Virus</th>
<th>pI</th>
<th>Structure</th>
<th>Shape</th>
<th>Envelope</th>
<th>Transmission route</th>
<th>Host</th>
</tr>
</thead>
<tbody>
<tr>
<td>MS2</td>
<td>3.9a</td>
<td>T3</td>
<td>Icosahedral</td>
<td>No</td>
<td>Fecal-oral</td>
<td>E. coli</td>
</tr>
<tr>
<td>FCV</td>
<td>4.9b</td>
<td>T3</td>
<td>Icosahedral</td>
<td>No</td>
<td>Respiratory</td>
<td>Feline</td>
</tr>
<tr>
<td>Echovirus 11</td>
<td>5.9c</td>
<td>Pseudo-T3</td>
<td>Icosahedral</td>
<td>No</td>
<td>Fecal-oral</td>
<td>Human</td>
</tr>
<tr>
<td>φX174</td>
<td>6.6d</td>
<td>T1</td>
<td>Icosahedral</td>
<td>No</td>
<td>Fecal-oral</td>
<td>E. coli</td>
</tr>
</tbody>
</table>

* From Ackermann and Michael (1).
* Obtained from http://www.embl-heidelberg.de/cgi/pi-wrapper.pl.

MATERIALS AND METHODS

Viruses and cells. To study the influence of the pI on virus attachment patterns, we chose viruses of varying pIs that included bacteriophages and known enteric and mammalian viruses (Table 1). Bacteriophage MS2 (ATCC 15597-B1) was propagated in *Escherichia coli* HS(pFamp)R (ATCC 700891). Bacteriophage φX174 (ATCC 13706-B1) was grown in *E. coli* host CN13 (ATCC 700609). The MS2 and φX174 phages were enumerated with the double agar layer method using their respective hosts. Echovirus 11 (ATCC VR-1052) was propagated in buffalo green monkey cells. The FCV strain P9 was grown in Crandell-Reese feline kidney (CRFK) cells. (The virus and host were a generous gift from Dr. Sagar Goyal at the University of Minnesota.) The mammalian viruses were enumerated using a soft agar overlay method. Propagation and maintenance media were identical to those of Gulati et al. (13), with the exception of lactalbumin hydrolysate. Lactalbumin hydrolysate was not used for CRFK cells. Buffalo green monkey cells were grown and maintained in media similar to CRFK cells, with the exception of 25 mM of N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid and 10% fetal bovine serum.

Attachment studies. Butterhead lettuce was purchased locally from a farmers’ market that sold fresh farm products. The lettuce leaves were severed from the base and cut into 25-cm² pieces with appropriate sterile techniques. The pieces were placed in a 20-oz Whirl-Pak bag (Fort Atkinson, Wis.). Twenty milliliters of citric phosphate buffer (0.1 M) (pH range, 3 to 5) was added to appropriately labeled bags. Twenty milliliters of sodium phosphate buffer (0.1 M) (pH range, 6 to 8) was similarly added to labeled bags. The buffers were titrated to their respective pH with HCl or NaOH as necessary, with an acceptable pH buffer variation of ±0.01 pH units. (The pH meter was calibrated with pH 4, 7, and 10 calibration buffers before each use.) The pH range was specifically chosen to include the pIs of the viruses being studied. A virus suspension (0.1 ml of a 10⁵ PFU/ml) was added to each bag to achieve a total of 10⁴ PFU per bag. The control treatments consisted of the bag that contained 20 ml of buffer inoculated with 0.1 ml of a 10⁵ PFU/ml lysate diluted in 0.1% peptone but without the piece of lettuce. The virus suspensions and the buffers used in the experimental and control treatments were prepared from the same stock. After the virus was inoculated into the control and experimental units, virus adsorption was allowed to proceed for 30 min at room temperature on a rocking platform. The experimental and control units were sampled in a staggered fashion. After 30 min, the control and experimental units were cut open using flame-disinfected scissors, and 0.1 ml of buffer was aspirated from the bags. The bacteriophage assays were performed immediately after each bag was cut open, since their assay was rather straightforward. The 0.1-ml aliquots were assayed for MS2 and φX174 bacteriophages without any further dilution. The aliquots for echovirus and FCV analysis were first diluted into Eagle’s minimal essential media (catalog no. M0643) (Sigma-Aldrich, St. Louis, Mo.). From the diluted buffer, 0.2 ml was inoculated into each well for the plaque assay. The animal virus samples were collected and then assayed together once the adsorption phase of the experiment was complete. To avoid experimental variability, buffer volumes, dimensions of the lettuce squares, sequence of addition of viruses and lettuce squares, and viral stocks were all standardized to the maximum extent possible.

Data analysis. Multiple, independent experimental trials, with each trial consisting of three experimental replicates and three control replicates, were performed. The samples from the control and experimental treatments were assayed at the same time. The percent attachment for each virus was calculated as follows: [(mean control – mean experimental)/mean control] × 100. We measured attachment as a function of a “difference,” because relying on virus titers based directly on extraction or removal from the leaf surface was prone to significant errors, since it was impossible to choose a buffer that guaranteed high recovery efficiency for the different viruses. Potential virus aggregation and deggregation and attachment to the bag were controlled by analyzing the data in terms of the experimental bag (with lettuce) to the control bag (without the lettuce). The data are presented as percent attachment with the trend line (based on the median value) along with the sample interquartile range (25th and 75th percentiles). Significant differences (P ≤ 0.05), if any in the percent attachment across the pH range for a given virus, were calculated based on the Wilcoxon sign rank test using SPSS statistical software, version 11.0.1 (SPSS Inc., Chicago, Ill.).

RESULTS

The attachment of enteric viruses and FCV to lettuce is shown in Figure 1A through 1D. The trend line that connected the median values along with the sample interquartile range (25th and 75th percentiles) is shown. The MS2 phage exhibited the most significant change in adsorption as a function of pH compared with the other three viruses (Fig. 1A). At pH 3.0, MS2 phage showed the maximum adsorption (28%, which corresponded to 2,690 PFU/25 cm²). At pH 8.0, most viruses were unattached (~32%). The FCV showed maximal attachment at pH 8.0 (19%, corresponding to 1,860 PFU/25 cm²) and minimal attachment at pH 5.0 (Fig. 1B). The FCV was rapidly inactivated at pH 3.0; hence, only data from pH 4.0 and above are presented. Although the FCV results indicate that there was significant adsorption and desorption of viruses at all of the pH ranges that were tested, it is apparent from the trend line that as the pH increased from 5.0 to 8.0, the percentage of viruses that were attached to the lettuce surface increased significantly (Table 2). An equal proportion of viruses ap-
FIGURE 1. Percent attachment of four different viruses to 25-cm² lettuce pieces in five different pH buffers. The trend line connects the median values. The horizontal bars represent the 25th and 75th percentiles. The vertical line represents the virus isoelectric point (pI). n represents the number of independent experimental trials with each trial having three replicates. (A) MS2 bacteriophage attachment to butterhead lettuce (n = 6). (B) Feline calicivirus (FCV) attachment to butterhead lettuce (n = 10 for pH 4, 6, and 8 experiments) (n = 9 for pH 5 and 7 experiments). (C) Echovirus 11 attachment to butterhead lettuce (n = 6 for pH 3, 4, and 5 experiments; n = 5 for pH 6 and 8 experiments; n = 7 for pH 7 experiments). (D) Bacteriophage φX174 attachment to butterhead lettuce (n = 6 for all experiments).

peared to be attached and unattached at pH 6.0 (Fig. 1B). It must be noted that the difference in attachment of FCV at pH 5.0 compared with pH 4.0 was only 3.4% (which is equivalent to 340 PFU/25 cm²).

Echovirus 11 exhibited the greatest attachment to butterhead lettuce among the four different viruses that were used in this study (Fig. 1C). The virus appeared to be strongly bound to the lettuce surface at pH 3.0 (14.4%, corresponding to 1,440 PFU/25 cm²) and tended to increase with increasing pH. Maximal attachment was observed at pH 8.0 (57% or 5,700 PFU/cm²).

The phage φX174 exhibited the most complex attachment and detachment pattern of the four viruses that were studied (Fig. 1D). At the extreme ends of the pH range
TABLE 2. P values obtained when comparing percent attachment of four different viruses to lettuce in five different pH buffers

<table>
<thead>
<tr>
<th>pH</th>
<th>Virus</th>
<th>4.0</th>
<th>5.0</th>
<th>6.0</th>
<th>7.0</th>
<th>8.0</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.0</td>
<td>MS2</td>
<td>0.03*</td>
<td>0.03*</td>
<td>0.03*</td>
<td>0.03*</td>
<td>0.12</td>
</tr>
<tr>
<td></td>
<td>φX174</td>
<td>0.04*</td>
<td>0.08</td>
<td>0.08</td>
<td>0.25</td>
<td>0.75</td>
</tr>
<tr>
<td></td>
<td>Echovirus 11</td>
<td>0.25</td>
<td>0.75</td>
<td>0.04*</td>
<td>0.05*</td>
<td>0.04*</td>
</tr>
<tr>
<td>4.0</td>
<td>MS2</td>
<td>0.75</td>
<td>0.75</td>
<td>0.46</td>
<td>0.17</td>
<td>0.08</td>
</tr>
<tr>
<td></td>
<td>φX174</td>
<td>0.60</td>
<td>0.75</td>
<td>0.24</td>
<td>0.01*</td>
<td>0.01*</td>
</tr>
<tr>
<td></td>
<td>FCV</td>
<td>0.68</td>
<td>0.24</td>
<td>0.12</td>
<td>0.08</td>
<td>0.01*</td>
</tr>
<tr>
<td></td>
<td>Echovirus 11</td>
<td>0.75</td>
<td>0.04*</td>
<td>0.12</td>
<td>0.08</td>
<td>0.01*</td>
</tr>
<tr>
<td>5.0</td>
<td>MS2</td>
<td>0.92</td>
<td>0.75</td>
<td>0.46</td>
<td>0.17</td>
<td>0.08</td>
</tr>
<tr>
<td></td>
<td>φX174</td>
<td>0.35</td>
<td>0.92</td>
<td>0.46</td>
<td>0.17</td>
<td>0.08</td>
</tr>
<tr>
<td></td>
<td>FCV</td>
<td>0.01*</td>
<td>0.01*</td>
<td>0.01*</td>
<td>0.01*</td>
<td>0.01*</td>
</tr>
<tr>
<td></td>
<td>Echovirus 11</td>
<td>0.08</td>
<td>0.12</td>
<td>0.08</td>
<td>0.08</td>
<td>0.01*</td>
</tr>
<tr>
<td>6.0</td>
<td>MS2</td>
<td>0.46</td>
<td>0.60</td>
<td>0.05*</td>
<td>0.05*</td>
<td>0.05*</td>
</tr>
<tr>
<td></td>
<td>φX174</td>
<td>0.60</td>
<td>0.46</td>
<td>0.05*</td>
<td>0.05*</td>
<td>0.05*</td>
</tr>
<tr>
<td></td>
<td>FCV</td>
<td>0.03*</td>
<td>0.01*</td>
<td>0.01*</td>
<td>0.01*</td>
<td>0.01*</td>
</tr>
<tr>
<td></td>
<td>Echovirus 11</td>
<td>0.50</td>
<td>0.46</td>
<td>0.05*</td>
<td>0.05*</td>
<td>0.05*</td>
</tr>
<tr>
<td>7.0</td>
<td>MS2</td>
<td>0.75</td>
<td>0.05*</td>
<td>0.08</td>
<td>0.08</td>
<td>0.08</td>
</tr>
<tr>
<td></td>
<td>φX174</td>
<td>0.05*</td>
<td>0.08</td>
<td>0.08</td>
<td>0.08</td>
<td>0.08</td>
</tr>
<tr>
<td></td>
<td>FCV</td>
<td>0.95</td>
<td>0.95</td>
<td>0.95</td>
<td>0.95</td>
<td>0.95</td>
</tr>
<tr>
<td></td>
<td>Echovirus 11</td>
<td>0.22</td>
<td>0.22</td>
<td>0.22</td>
<td>0.22</td>
<td>0.22</td>
</tr>
</tbody>
</table>

* Numbers with asterisks represent significant differences based on the Wilcoxin sign rank test (P < 0.05).

b The FCV was nonviable at pH 3.0.

tested (pH 3.0 and 8.0), φX174 phage exhibited only 4.9 and 6% (490 and 600 PFU/25 cm², respectively) attachment, respectively. There appeared to be no attachment of virus particles to the leaf surfaces between these two pH values.

DISCUSSION

The physicochemical forces that control the interaction between enteric viruses and plant surfaces have not been adequately studied in the past. A better understanding of the controlling factors can lead to improved washing or other intervention strategies. The use of positively charged filters is a common strategy used to concentrate enteric viruses in suspension. The apparent reversible and irreversible attachment that was observed in these studies (based on the data spread) was expected, because these viruses do not use specific cell surface receptors to attach to the lettuce surface.

In batch experiments using sediment material, virus concentrations (in the buffer) normally decline in time (due to adsorption onto the solid surface) after which they remain constant. When the numbers remain constant, the viruses are said to be at equilibrium adsorption, which is achieved due to reversible adsorption (4, 21). It has been postulated that two processes are involved in the formation of the adsorption equilibrium with a solid surface, namely, mass transport of viruses close to the surface and the immobilization of the viruses to the surfaces by physical and chemical interactions. Electrostatic interactions, van der Waals forces, and hydrophobic effects are three major forces that are thought to be responsible for the interactions between virus particles and solid substrates (21). The Der-jaguin-Landau-Verway-Overbeek (DLVO) theory serves as a conceptual framework to understand nonspecific interaction of virus particles to solid surfaces under different conditions such as pH and ionic strength. The DLVO theory states that only the van der Waals and electrostatic forces are of any consequence in colloidal particle adhesion to surfaces. The van der Waals force is always attractive, and the electrostatic force is always repulsive. Factors that decrease or increase the electrostatic component will invariably reduce or enhance the influence of van der Waals force, thereby directly affecting adsorption. Even though hydrophobic interactions are thought to be involved in virus adsorption to solid surfaces, hydrophobic interactions are not considered in the DLVO theory. Previous studies have shown that virus attachment to solid surfaces decreases with increasing pH (11, 21). The DLVO theory attempts to explain this phenomenon by suggesting increased repulsion by negatively charged virus particles and solid surfaces. The overall negative charge on the virus surfaces increases above the pI of the virus particle, whereas below the viral pI the overall net charge becomes increasingly positive. Even though the surface charge of the lettuce is relatively unknown (to the best of our knowledge), the experimental design normalized the impact of the lettuce charges across all experiments. Among the four viruses that were studied, only the MS2 phage behaved per the DLVO theory (Fig. 1A). As the pH increased from 3.0 to 8.0, the percentage of virus attachment decreased. The enteric virus echovirus 11 exhibited a pattern of increasing attachment (Fig. 1C). Below the pI of the virus (i.e., 5.9) the viruses were “tightly” bound to the leaf compared with “reversible” binding above the pI. (The reversible binding is deduced by the spread of the data points at pH levels greater than the pI.)
Guan et al. (12) recently reported the existence of a “critical pH” range at which the virus behavior changes abruptly. The critical pH was reported to be 0.5 pH units below the highest pI of the virus and the solid substrate. If the pH of the suspending medium is below the critical pH, they suggest that the virus has an opposite charge to at least one component of the solid substrate and thus becomes irreversibly adsorbed to the substrate. The critical pH concept, however, based on this study does not appear to be valid for any virus other than MS2 phage. The phage φX174 exhibited a completely different adsorption pattern compared with the other three viruses, with attachment occurring only at the extreme pH levels.

Previous studies that involved hepatitis A virus (HAV) and poliovirus recovery from fruits and vegetables showed variable efficiencies, suggesting that the detachment process is a net result of complex virus particle-surface interactions (14, 27). Studies by Legitt et al. (14) and Ward et al. (27) used a high pH (9.0) buffer wash to recover viruses from lettuce. Legitt et al. recovered only 16% of inoculated poliovirus and HAV from lettuce. Ward et al. recovered 58 and 49% of poliovirus from 2.9 and 3.3 kg of lettuce, respectively, suggesting poliovirus adsorption to lettuce. Most virus particles were therefore being “lost” after the first pH 9.0 wash. Furthermore, the results seem to suggest that the greater the amount of lettuce used, the greater was the “loss” of virus particles. Our study did not use poliovirus or HAV; it used echovirus 11, a member of the picornavirus family (similar to poliovirus and HAV). However, we hypothesize that many enteric picornaviruses share similar adsorption potential to fruits and vegetables. The low recoveries of echovirus in the present study similar to the low recoveries observed by Legitt et al. support this hypothesis. The increased attachments of echovirus 11 to lettuce as a function of increasing pH suggest the involvement of electrostatic forces. However, other investigators have suggested that hydrophobic interactions may also be playing a key role in virus attachment (15, 22).

The FCV is routinely used as a surrogate for norovirus, since these two viruses are genetically and structurally related (2, 13, 23). The FCV infects the respiratory tract; therefore, little evolutionary pressure exists to maintain capsid integrity at low pH. The virus is sensitive to pH 3.0 as observed in this study. Duizer et al. (8) also reported that less than 0.005% of FCV F9 virus survived at pH 3.0 after 30 min. The results of the present study suggest that FCV is not a suitable surrogate for low pH studies. Nevertheless, in this study FCV was able to bind to lettuce at pH 7.0 and 8.0. The FCV was able to bind to butterhead lettuce at approximately 18.6 or 1,860 PFU/25 cm² at neutral to basic pH. The ramifications of these results, however, have to be considered carefully, since the transmission route of FCV is different from that of the typical enteric norovirus. It is plausible that the attachment patterns of norovirus may actually be closer to that of echovirus 11 rather than FCV, since noroviruses and echovirus 11 are enteric viruses.

Overall, these results suggest that different viruses can exhibit differing attachment patterns to lettuce. Importantly, unlike the situation with aquifer sediments, the pI of the virus is not the controlling factor that governs their attachment to lettuce. This study is significant in that it demonstrates that the attachment of enteric virus particles to lettuce cannot be generalized from the pattern of a single commonly used enteric virus surrogate such as the MS2 or φX174. These results suggest that current fruit and vegetable washing and rinsing protocols to rinse or recover viruses need be reexamined, because pH interactions among the virus particle, the surface, and the buffer can alter the attachment or detachment of the viruses to the surfaces. The study also suggests that use of a particular pH buffer to recover virus particles could artificially select for the recovery of a particular type or group of viruses. This may be the reason why in an earlier study we recovered only DNA-containing phages from cilantro and parsley samples (9).

ACKNOWLEDGMENTS

E. Vega was supported by a National Aeronautics and Space Administration Graduate Student Research Fellowship (project NGT10-52628). The U.S. Department of Agriculture (USDA), Initiative for Future Agriculture and Food Systems (I4AFS) project USDA-CSREES (Cooperative State Research, Education, and Extension Service)–I4AFS program (00-52102-9637) and Hatch grant (H8708) also supported parts of this study. Dr. Goyal is gratefully acknowledged for providing CRFK cells and FCV.

REFERENCES


