Stability of bovine coronavirus on lettuce surfaces under household refrigeration conditions

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ARTICLE INFO

ABSTRACT

Fecal suspensions with an aerosol route of transmission were responsible for a cluster of severe acute respiratory syndrome (SARS) cases in 2003 in Hong Kong. Based on that event, the World Health Organization recommended that research be implemented to define modes of transmission of SARS coronavirus through sewage, feces, food and water. Environmental studies have shown that animal coronaviruses remain infectious in water and sewage for up to a year depending on the temperature and humidity. In this study, we examined coronavirus stability on lettuce surfaces. A cell culture adapted bovine coronavirus, diluted in growth media or in bovine fecal suspensions to simulate fecal contamination was used to spike romaine lettuce. qRT-PCR detected viral RNA copy number ranging from 6.6×10^4 to 1.7×10^6 throughout the experimental period of 30 days. Whereas infectious viruses were detected for at least 14 days, the amount of infectious virus varied, depending upon the diluent used for spiking the lettuce. UV and confocal microscopic observation indicated attachment of residual labeled virions to the lettuce surface after the elution procedure, suggesting that rates of inactivation or detection of the virus may be underestimated. Thus, it is possible that contaminated vegetables may be potential vehicles for coronavirus zoonotic transmission to humans.

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1. Introduction

Coronavirus (CoV) is an enveloped, single-stranded, positive-sense RNA virus. Although respiratory CoVs are largely secreted by the nasal route, the role of fecal/oral transmission has not been fully explored. Earlier studies have demonstrated that CoV stability largely depends on environmental temperature (Ijaz et al., 1985; Siddell et al., 1983; Tennant et al., 1994). CoVs are inactivated at 56 °C within 10–15 min, at 37 °C after several days, and at 4 °C after several months. However, at −60 °C, CoVs can survive for many years without loss of infectivity (Andries et al., 1978; McIntosh et al., 1974; Siddell et al., 1983). Survival studies of human coronavirus (HCoV)-229E and HCoV-OC43 demonstrated that both viruses survived in a saline solution up to 6 days at room temperature, but after drying on hospital surfaces, they survived as long as 3 h; although survival differences between the two viruses were observed (Sizun et al., 2000). These findings suggest that surfaces and suspensions can be sources of contamination for hospital-acquired CoV infections (Sizun et al., 2000).

During the Severe Acute Respiratory Syndrome (SARS) epidemic in Hong Kong, it was thought that aerosolized droplets of feces were responsible for the transmission of a cluster of 420 SARS cases (McKinney et al., 2006). Consequently, the World Health Organization (WHO) urged that research be undertaken to better define the modes of transmission of SARS-CoV through sewage, feces, food and water (WHO, 2003). A more recent WHO report has listed hepatitis E virus (HEV), Highly Pathogenic Avian Influenza (HPAI) virus H5N1, SARS-CoV and Nipah virus as having the potential for foodborne transmission. Initial foodborne transmission is a route to enter the human population, which can then shift and spread through human-to-human transmission. Furthermore, virus long-term survival would not be a requirement when contamination occurs at the end of the food processing chain or in fresh produce (FAO/WHO, 2008). The risks of contamination of agricultural fields and field workers by animal enteric/respiratory CoV cannot be ignored. Furthermore, in times of outbreaks food handlers may also be responsible for food contamination with CoV. Because human enteric coronavirus causes a mild disease of lower

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incidence, foodborne transmission of CoV has not compelled an investigation.

A recent environmental survival study of two animal CoVs demonstrated that these viruses remained infectious in water and sewage at 27 °C for periods of weeks and at 4 °C for up to a year (Casanova et al., 2009). However, the role of ready-to-consume produce as a vehicle for potential zoonotic transmission or a source for genetic recombination during co-infections between animal and human CoVs has not yet been investigated.

The potential zoonotic transmission of CoVs was first suggested by studies in which human enteric coronavirus (HECoV)-4408 isolated from a diarrheic child was found to be indistinguishable genetically, antigenically and cytopathogenically from bovine CoV (BCoV) (Zhang et al., 1994) and retained infectivity and pathogenicity when inoculated into gnotobiotic calves (Han et al., 2006). In addition, BCoV is evolutionarily closely related to HCoV-OC43 (Vijgen et al., 2005) and to a lesser extent to SARS-CoV (Zhang et al., 2005). Therefore, in the present study, we used BCoV strain 88 as a model to examine the stability and the potential for foodborne transmission of CoV.

2. Materials and methods

2.1. Virus spiking

A total of 10 Romaine lettuce heads were purchased in separate occasions from local markets and used immediately. Undamaged leaves were chosen for use in the experiments. Prior to use, the leaves were washed twice in deionized water; the water was drained and the leaves were air-dried. Leaves were surface-sterilized with UV light for 20 min on each side and then cut into 4×4 cm sections. The cell adapted bovine coronavirus strain 88 (BCoV-88) was propagated in human rectal tumor (HRT)-18 cells, diluted to a final concentration of 1.2×10^5 plaque forming units (PFU) in either minimum essential medium (MEM) or a bovine fecal suspension. Two bovine fecal suspensions (0.1% and 10%) were used to dilute the virus, representing light and heavy fecal contaminations (1 ppm and 100 ppm), respectively. Because enteric viruses are shed in titers up to 10^{11} particles per gram of feces, the virus was diluted to simulate 1 ppm of viral contamination. Each piece of prepared lettuce was spiked with 100 μl of the virus preparation, allowed to dry for 2 h in a biosafety cabinet and then stored at 4 °C in a refrigerator until elution. Bovine feces were obtained from a healthy cow, resuspended at 0.1% and 10% in minimum essential medium (MEM), centrifuged at $3000 \times g$ and filtered through a 0.2 μM syringe filter. The suspensions were confirmed to be BCoV negative by qRT-PCR before being used, as described later. Experiments were duplicated using feces from a healthy young calf, also confirmed negative for BCoV by qRT-PCR.

2.2. Virus elution

To establish an optimal elution method, a pilot procedure was conducted on day 0. Virus from triplicate lettuce pieces was eluted with MEM + 2% fetal bovine serum (FBS, Gibco), Tris-glycine + 1% FBS or phosphate-buffered saline (PBS)-Triton X-100 + 0.5% FBS, immediately following the drying step. The eluents were then precipitated with 10% polyethylene glycol (PEG) 6000 (Calbiochem, EMD Biosciences, La Jolla, CA) and 2.5% NaCl at 4 °C with agitation for 2 h followed by centrifugation at $3500 \times g$ for 30 min at 4 °C. The pellet was reconstituted with MEM + 2% FBS and subsequently analyzed by qRT-PCR for detecting viral genomic RNA. Similar results were obtained with both MEM + 2% FBS and Tris-glycine + 1% FBS elution buffers. However, significantly lower viral RNA copy numbers were detected when the elution buffer

containing Triton X-100 was used (data not shown). Because MEM + 2% FBS would interfere less with an infectivity assay than the buffer containing Tris-glycine, MEM + 2% FBS was selected as the eluent in subsequent experiments. Virus was eluted in triplicate samples on days 0, 2, 5, 7, 12, 14, 20, 26 and 30. Twenty milliliters of elution buffer was added to each lettuce piece in a 50 ml conical tube which was agitated for 15 min on an orbital shaker at 100 rpm at room temperature. Virus was then precipitated with 10% PEG 6000 and 2.5% NaCl, as described. The pellet was reconstituted with the elution buffer (MEM + 2% FBS) to 250 μl and stored at −70 °C. Experiments were repeated on three different occasions, with 9 samples per time point for each group. To estimate the amount of virus particles lost during drying and elution, each virus dilution was kept at 4 °C, sampled in triplicate at each time point, precipitated and reconstituted in the same way as the lettuce spiked virus.

2.3. Viral RNA extraction and qRT-PCR

Viral RNA was extracted from 90 μl of resuspended pellet using the MagMax™ viral RNA isolation kit and the MagMAX™ Express Magnetic Particle Processor (Applied Biosystems/Ambion, Austin, TX). Extracted RNA samples were either analyzed immediately by qRT-PCR or stored at −80 °C until use. For qRT-PCR, we used primers and a Locked Nucleic Acid (LNA) Cy5 labeled fluorescent probe (Integrated DNA Technology, Coralville, IA) for the open reading frame (ORF) 1b region of CoV genomic RNA (Escutenaire et al., 2007; Muradrasoli et al., 2009). A synthetic oligonucleotide complementary to the probe was used to generate a standard curve (Escutenaire et al., 2007). Primers and probes for human 18S RNA (Cat# 4308329, Applied Biosystems, Foster City, CA) were used as internal controls (Poon et al., 2004). BCoV-88 RNA was used as a positive control. RNA from mock infected cell supernatant was used as a negative control and RNAse-free water was used as a non-template control. For all assays, 8 μl of RNA was transferred to a Qiagen Rotor-Gene strip tube containing 12 μl of the Rotor Gene™ Multiplex qRT-PCR mix (Qiagen, Valencia, CA). Cycling conditions were 50 °C for 30 min, 95 °C for 10 min, 5 touchdown amplification steps of 94 °C for 30 s and 56 °C for 30 s, decreasing by 2 °C every second cycle down to 48 °C for 30 s, and then 50 cycles of 94 °C for 30 s and 46 °C for 60 s. Amplification was detected using a Rotor Gene Q 6 plex machine from Qiagen.

2.4. Virus plaque assay

A plaque assay (Hasoksuk et al., 2008) was used to quantify infectious virus recovered from the lettuce surface. Briefly, 6-well plates containing 3-to 5-day-old monolayers of HRT-18 cells were rinsed and incubated with FBS-free MEM for 3 h at 37 °C in a 5% CO₂ atmosphere. The culture medium was then aspirated and serial dilutions of the recovered virus preparations from the lettuce leaves were added to duplicate wells. Following virus adsorption for 1.5 h at 37 °C in a 5% CO₂ atmosphere, the inoculum was removed and the cell monolayer was rinsed with FBS-free MEM. The plates were then overlaid with MEM containing 0.8% Noble agar, 0.1% neutral red, 0.15 μg/ml of trypsin (Cat#1426 − Sigma, St. Louis, MO), 1% diethylaminoethyl dextran, 1% antibiotic-antimycotic (Gibco, Carlsbad, CA) and 3% sodium bicarbonate. Trypsin was added to the overlay media to mimic host cell proteases by activating viral spike protein, increasing coronavirus infectivity (Bertram et al., 2011; Gaertner et al., 1991; Holmes, 2001). After solidification of the agar, the plates were inverted and incubated at 37 °C. Plaques were counted from days 4–6. The final titers were calculated by averaging counted plaques on duplicate wells of the highest dilution.

2.5. Cell culture immunofluorescence (CCIF) assay

Because of the long period of incubation required for plaque formation in the plaque assay, cell toxicity was observed when the recovered virus was originally diluted in fecal suspensions, despite rinsing the monolayer with FBS-free MEM after adsorption. A cell culture immunofluorescence assay was used to circumvent this problem. Briefly, 3–4-day-old 96-well plates containing monolayers of HRT-18 cells were rinsed with FBS-free MEM and incubated for 3 h at 37 °C in a 5% CO_2 atmosphere. Serial dilutions of recovered virus were added to duplicate wells, followed by the addition of 0.04 μg trypsin per well. Plates were then centrifuged for 1 h at $500 \times g$ at room temperature and incubated at 37 °C, 5% CO_2 for 20 h. Plates were then fixed with 80% acetone in water, stained with guinea pig anti-BCoV antiserum (BEI Resources cat# NR-455) and FITC-conjugated goat anti-guinea pig IgG (KPL, Inc, Gaithersburg, MD), and observed using an inverted UV microscope. The final titers were calculated by averaging counted fluorescing cells in duplicate wells of the highest dilution of the virus where fluorescing cells were observed.

2.6. Presence of residual virus

To determine if residual virus would remain on the lettuce surface after the elution procedure, BCoV-88 was concentrated by ultracentrifugation onto a 20% sucrose cushion, labeled with SYBR-Gold (Invitrogen, Carlsbad, CA) and purified using a 20–60% continuous sucrose gradient at $105,000 \times g$ for 18 h using a Beckman SW41TI rotor. Labeled virus was diluted in MEM + 2% FBS, 0.1% fecal suspension or 10% fecal suspension and was spiked onto the lettuce. After drying, the virus was eluted from the lettuce surface as described. Lettuce pieces were then observed using a UV microscope and/or fluorescence confocal microscope for detection of residual virus on lettuce leaves.

2.7. Statistical analysis

Results from (log-transformed) qRT-PCR and infectivity assays were compared among and within the groups using two way ANOVA testing for group, time, and the interaction of group and time, using the SigmaStat® program (Systat Inc). Statistical significance was assessed at $p < 0.05$.

3. Results

3.1. Viral genomic RNA was detected from the lettuce surface after spiking and storage in a refrigerator

To determine the stability of the viral genomic RNA on the lettuce surface under household refrigeration conditions, we used qRT-PCR to detect and quantify viral RNA at various time points after storage of virus-spiked lettuce at 4 °C (Fig. 1A). Whereas viral RNA was detected from the lettuce surface at all time points (up to 30 days) throughout the experiment, RNA levels varied significantly among the different experimental groups (6.6×10^4 to 1.7×10^6 copy numbers). In general, viral RNA levels were significantly higher ($p < 0.05$) when the spiked virus was diluted with MEM + 2% FBS than when diluted with fecal suspensions (Fig. 1A). Analysis of variance within groups showed a significant decrease ($p < 0.05$) in the amount of RNA detected from lettuce after day 5 for MEM + 2% FBS and 0.1% fecal suspensions, and after day 12 for 10% fecal suspensions.

When the virus was stored in suspensions at 4 °C, there was no significant decrease in the amount of viral RNA detected
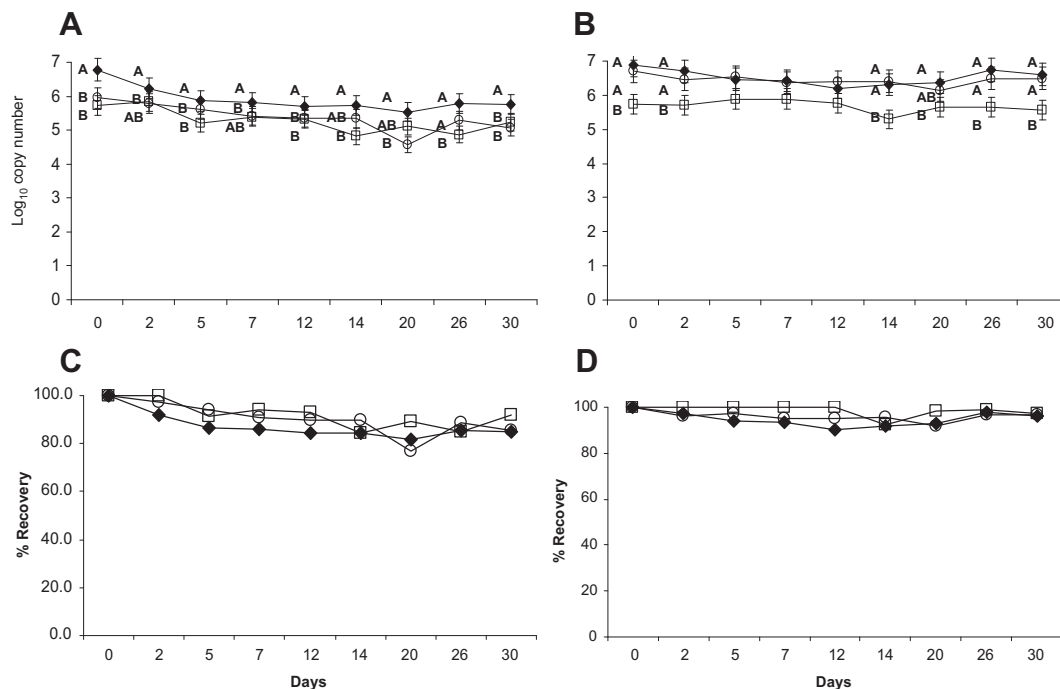

Fig. 1. Viral RNA recovered from spiked lettuce or from virus stored in suspension detected by real-time RT-PCR for coronavirus. A. Log_{10} of viral RNA copy number recovered from the lettuce surface over time after elution; B. Log_{10} of viral RNA copy number recovered from virus stored in suspension over time; C. Log_{10} of viral RNA copy number recovered from the lettuce surface over time after elution normalized to 100% at day 0; D. Log_{10} of viral RNA copy number recovered from virus stored in suspension over time normalized to 100% at day 0. Different letters denote statistically significant differences at $p < 0.05$ (Two way ANOVA using all pairwise multiple comparison procedures: Holm–Sidak method) among groups at same time point. Statistical symbols: A is statistically different from B at $p < 0.05$. Legend: -◆- CoV in MEM + 2% FBS; -○- CoV in 0.1% fecal suspension; -□- CoV in 10% fecal suspension.

throughout the 30 day experimental period between MEM + 2% FBS and 0.1% fecal suspension (Fig. 1B). However, the amount of viral RNA detected from the 10% fecal suspension was generally lower at all time points, and significantly lower ($p < 0.05$) at days 0, 2, 14, 20, 26 and 30, when compared to the 0.1% fecal suspension or MEM + 2% FBS treatment.

To better estimate long-term stability of coronavirus RNA, the recovery of viral RNA was expressed as a percentage of the log10 copy number of viral RNA revered at various time points relative to the viral RNA recovered at day 0 which was normalized to 100%. As shown in Fig. 1C and D, there was less than 20% reduction in viral RNA recovery from spiked lettuce or stored in suspension for 30 days. These results demonstrate that CoV RNA was stable on the lettuce surface and in the suspensions tested throughout the 30 days period.

3.2. Infectious virus was detected from lettuce after spiking and storage in a refrigerator

To assess the stability of infectious virus on the lettuce surface, we used a plaque assay to detect and quantify infectious virus recovered from lettuce after spiking and storage at 4 °C for various periods of time. The infectivity of recovered virus varied significantly depending upon the type of diluent used (Fig. 2A). Overall, the infectivity (PFU) was significantly higher at each time point for virus diluted in MEM + 2% FBS than for those in either of the two fecal suspensions. Specifically, virus infectivity from MEM + 2% FBS decreased gradually from approximately 10,000 PFU on day 0−10 PFU on day 20 and was below the detectable level by day 25,

whereas the CoV infectivity from the 0.1% fecal suspension decreased similarly until day 12, but was no longer detectable by day 14. In contrast, no infectious virus was recovered from lettuce spiked with virus diluted in a 10% fecal suspension from day 2 onward. When the virus was stored in MEM + 2% FBS or in 0.1% fecal suspension, the reduction of infectivity over 30 days was much slower than that on the lettuce surface, but it was similar on the lettuce surface and in the 10% fecal suspension (Fig. 2B). Virus infectivity did not change significantly in MEM + 2% FBS diluent from day 0 to day 25, decreasing slightly by day 30. When the number of plaque forming units was normalized to 100% recovery at day 0 (Fig. 2C and D), a 90% reduction (1 log) every 5−6 days was observed in infectious virus recovered from lettuce surfaces spiked with CoV in MEM + 2% FBS or 0.1% fecal suspension. A 90% reduction in recovery was observed every 14 days from virus stored in 0.1% fecal suspension, and 30 days if stored in MEM + 2% FBS. These results demonstrate that infectious virions are more stable in suspension than when dried on the lettuce surface and that viral stability is influenced by the type of diluent used in the study.

These results also suggest that the 10% fecal suspension might contain factors that can inactivate virus during storage, thus reducing infectivity, or inhibiting plaque formation during a plaque assay. To distinguish these two possibilities, we used an immunofluorescence assay to determine the fluorescence foci following infection of cell cultures with the recovered virus. We found that overall there were more focus forming units (FFU) than PFU detected by the plaque assay (Fig. 3 vs Fig. 2). However, significant amounts of infectious virus (∼3 $\log_{10}$ FFU) were detected by the cell culture fluorescence assay at day 14 in samples recovered from
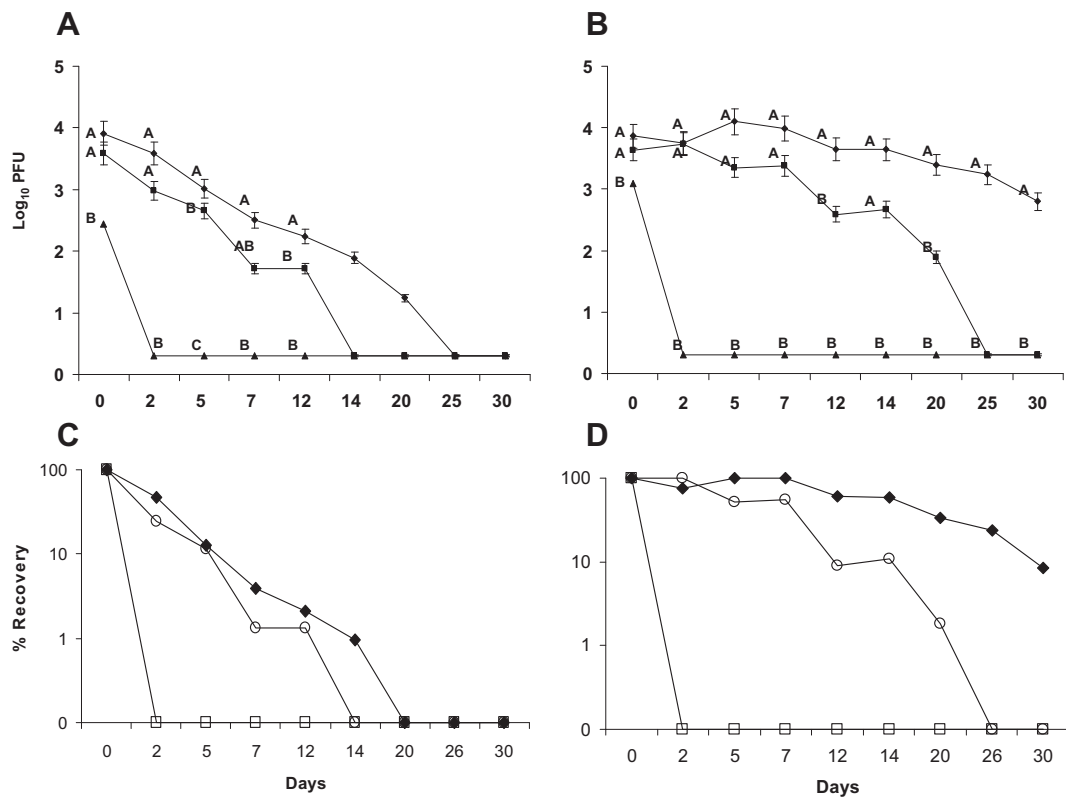

Fig. 2. Infectious coronavirus recovered from spiked lettuce or stored in suspensions, detected by plaque assay. A. Plaque forming unit (PFU) [$\log_{10}$] of infectious virus recovered from the lettuce surface over time after elution; B. PFU ($\log_{10}$) of infectious virus recovered from virus stored in suspensions over time; C. PFU [$\log_{10}$] of infectious virus recovered from the lettuce surface over time after elution normalized to 100% at day 0; B. PFU ($\log_{10}$) of infectious virus recovered from virus stored in suspensions over time normalized to 100% at day 0. Different letters denote statistically significant differences at $p < 0.05$ (Two way ANOVA using all pairwise multiple comparison procedures: Holm−Sidak method) among groups at same time point. Statistical symbols: A is statistically different from B and C; B is statistically different from C at $p < 0.05$. Legend: -◆- CoV in MEM + 2% FBS; -○- CoV in 0.1% fecal suspension; -□- CoV in 10% fecal suspension.
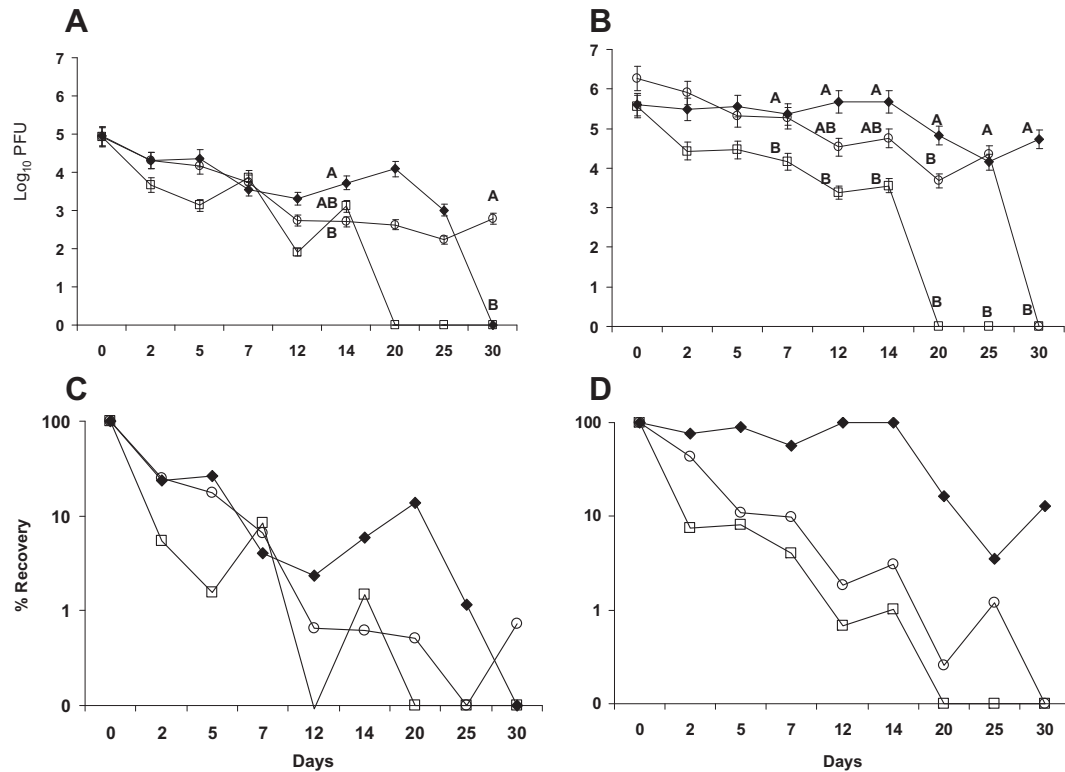
Fig. 3. Infectious coronavirus recovered from spiked lettuce or stored in suspensions and detected by cell culture immunofluorescence (CCIF) assay. A. Focus forming unit (FFU) ($\log_{10}$) of infectious virus recovered from the lettuce surface over time after elution; B. FFU ($\log_{10}$) of infectious virus, recovered from virus stored in suspensions over time; C. FFU ($\log_{10}$) of infectious virus recovered from the lettuce surface over time after elution normalized to 100% at day 0; D. FFU ($\log_{10}$) of infectious virus, recovered from virus stored in suspensions over time normalized to 100% at day 0. Different letters denote statistically significant differences at $p < 0.05$ (Two way ANOVA using all pairwise multiple comparison procedures: Holm−Sidak method) among groups at same time point. Statistical symbols: A is statistically different from B at $p < 0.05$. Legend: -◆- CoV in MEM + 2% FBS; -○- CoV in 0.1% fecal suspension; -□- CoV in 10% fecal suspension.

both lettuce spiked with virus diluted in 10% fecal suspension (Fig. 3) and the corresponding diluent, whereas the plaque assay resulted in no detectable levels of virus infectivity at day 2 (Fig. 2). When the number of fluorescent foci was normalized to 100% at day 0 (Fig. 3C), a 90% decrease in recovery was observed every 7 days for virus recovered from spiked lettuce of all three diluents. Similar observation was made from virus stored in 0.1 and 10% fecal suspensions (Fig. 3D), whereas reduction of 90% was only observed after 25 days for virus stored in MEM + 2% FBS. Combined with data in Figs. 2 and 3, these results indicate that viral infectivity was less stable in fecal suspensions than in MEM + 2% FBS and that an immunofluorescence assay may be more sensitive than the plaque assay for quantifying infectious virus. An immunofluorescence assay may be particularly useful with samples containing higher concentrations of feces that could interfere with plaque formation.

3.3. Residual coronavirus remained on the lettuce surface after elution procedure

To determine the efficiency of elution and to test the hypothesis that the degree of fecal contamination could affect the effectiveness of virus elution, we used SYBR-Gold to label virus particles, then diluted the labeled virus with one of three diluents and spiked the virus onto the lettuce surface. Following the same procedure for processing, storage and virus elution as described earlier, residual virus particles on the lettuce surface were subjected to microscopic examination. Results confirmed that residual coronavirus was present on the lettuce surface after elution (Fig. 4) with increased virus accumulation on the lettuce veins (Fig. 4E−G). It appears that

slightly more residual virus remained on the lettuce surface when the spiked virus was diluted in fecal suspensions (Comparing panels C and D with B in Fig. 4).

4. Discussion

In this study, we tested the hypothesis that contaminated vegetables may serve as a vehicle for coronavirus transmission to humans through consumption of produce. Specifically we used BCoV as a surrogate of the Genus *Betacoronavirus*, which includes SARS-CoV, to evaluate the stability of coronaviruses on the lettuce surface under household refrigeration conditions. Our data showed that BCoV on lettuce retained infectivity for at least 14 days. We also observed that higher numbers of infectious particles were detected using fluorescent focus forming assay compared to plaque assay. Plaque assay relies on the induction of cell death to form plaques and is sensitive to changes in the assay condition, whereas fluorescent focus assay relies on the detection of virus antigen within infected cells, which may increase the detection and visualization of the infectious virus particles (Flint et al., 2009). Interestingly, as assessed by a plaque assay, significantly lower amounts of infectious virus were recovered from lettuce surfaces spiked with virus samples that were diluted in a 10% fecal suspension as compared to those diluted in MEM + 2% FBS and a 0.1% fecal suspension (Fig. 2). The reason for such a rapid reduction in infectivity in virus diluted in 10% fecal suspensions is not known. It is possible that the high fecal concentration contains inhibitory factors, such as intestinal proteases or lipases. The abundant proteolytic or lipolytic enzymes present in the intestine, including those from bacterial flora, are
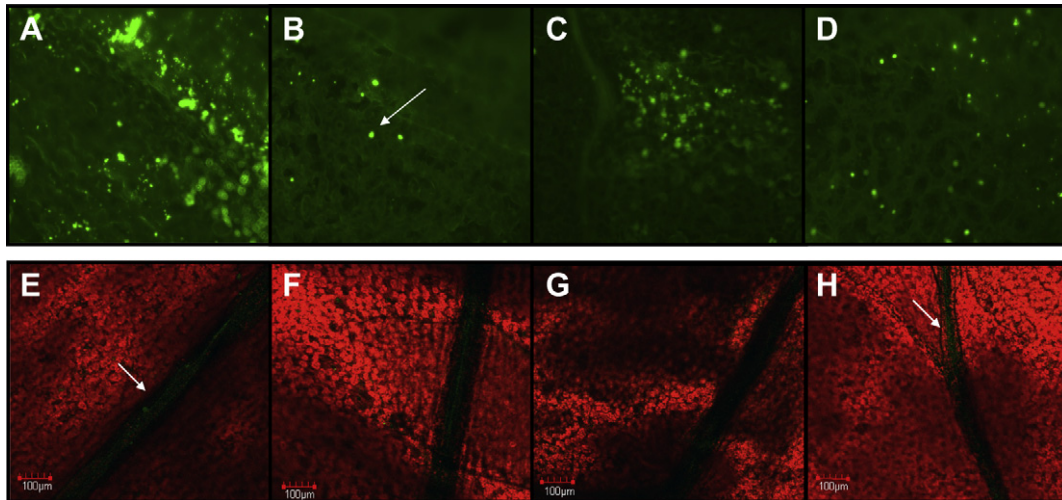
Fig. 4. Residual SYBR-Gold labeled coronavirus on the lettuce surface after elution with MEM + 2% FBS. A–D images were acquired using a Nikon Eclipse TS100 UV microscope using the Image NIS element program with a 40× objective. E–H images are confocal micrographs using an Olympus Fluoview™ FV 1000 confocal microscope. The laser setting was the excitation filter DM 488 and the Emission filters BA (505–525 nm) and BA (560–620 nm). The objective setting was 10× in the image; 100 μm marker bars are shown. A, E. Lettuce leaf spiked with CoV SYBR-Gold labeled, diluted in MEM + 2% FBS before elution; B, F. Lettuce leaf spiked with CoV SYBR-Gold labeled diluted, in MEM + 2% FBS after elution C, G. Lettuce leaf spiked with CoV SYBR-Gold labeled, diluted in 0.1% fecal suspension after elution and D, H. Lettuce leaf spiked with CoV SYBR-gold labeled diluted in 10% fecal suspension after elution. Arrows indicate labeled virus particles.

required for digestion of food and may destroy the virion surface spike glycoprotein, which is sensitive to protease cleavage and the virus envelope, which is sensitive to lipases (Chen, 1985; Krempl et al., 2000). Thus, the proteolytic and lipolytic effects of fecal suspensions might render BCoV less infectious. A similar finding has been reported for canine CoV: at 4 °C, fecal dilutions of 1:1 resulted in a rapid loss of viral infectivity, but a fecal dilution of 1:1000 preserved infectivity (Tennant et al., 1994). However, viral genomic RNA levels were more stable and steady on the lettuce surface for the duration of the experiment, compared to infectious virus. This suggests that the rapid decrease in CoV infectivity in the 10% fecal suspension was not due mainly to degradation of the viral genome, but more likely due to the loss of a functional spike glycoprotein on the virion surface that confers infectivity or to the loss of the virus envelope. However, neither the structure of the spike glycoprotein, nor the integrity of the virions or the viral genomic RNA recovered from the lettuce surface was determined in this study.

Microscopic observation of labeled virus on the lettuce surface indicates that some residual virus particles remained attached after elution, suggesting that virus elution was incomplete (Fig. 4). A quantitative assay to enumerate virus particles on the lettuce surface after elution may be required to establish if the differences in infectivity among the three diluents are the result of time-related inactivation or time-increased attachment. However, qRT-PCR results for detecting viral genomic RNA after virus elution from the lettuce surface support the former possibility because the difference in viral genome copies among the three diluents was lower than that for CoV titers in viral infectivity assay and remained relatively constant throughout the 30 days (comparing Figs. 1 and 2). It is not clear, however, whether the lower levels of viral genomic RNA, especially in the 10% fecal suspensions after elution (Fig. 1) resulted from RNA degradation or incomplete elution (Fig. 4). For non-enveloped viruses, the presence of biosolids increased viral attachment to the lettuce surface (Wei et al., 2009). Adsorption of non-enveloped virus particles may be linked to the virus adsorbent isoelectric point, hydrophobicity and ionic strength (Charles et al., 2008). Similar studies are needed for enveloped viruses and also to establish the role of biosolids on coronavirus environmental maintenance and survival.

Our results demonstrate that coronavirus was stable during the shelf-life of romaine lettuce, and that a wash procedure (elution) did not completely remove residual viruses. Thus, it is possible that contaminated ready-to-consume produce may be a potential vehicle for zoonotic transmission of coronaviruses to humans. However, the epidemiologic significance of the amount of recovered infectious particles cannot be evaluated, since the coronavirus infectious dose is unknown. Developing countries have an increasing role in food production for the world market thus increasing the necessity of monitoring food production beyond our borders (Newell et al., 2010). Although the probability of foodborne transmission of certain newly emerging viruses may be low, this transmission route may cause higher rates of disease and morbidity (FAO/WHO, 2008). Awareness regarding the possible roles of water, fresh produce and fecal contamination in coronavirus transmission is required at times of human coronavirus outbreaks.

Several coronaviruses can replicate in the epithelial cells of the respiratory tract as well as the enteric tract, and they can be transmitted by the respiratory route (Holmes, 2001). Bovine coronavirus has been described as a pneumoenteric virus that infects the upper and lower respiratory tract and intestine of cattle and wild ruminants. It is shed in feces and nasal secretions, causing respiratory and enteric syndromes such as shipping fever and winter dysentery in cattle (Saif, 2010). During the SARS outbreak, diarrhea was a common symptom of SARS, occurring in approximately 38% of the patients, demonstrating that SARS also had an intestinal tropism (Leung et al., 2003). However, foodborne transmission of SARS Coronavirus was not documented during the SARS outbreak and oral-fecal transmission of SARS is yet to be explored.

Disclaimer

Acknowledgements

Guinea Pig), NR-455. Part of this study was conducted in the Nanotechnology Core Facility (NanoCore) at the National Center for Toxicological Research, US Food and Drug Administration.

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