Prevalence of human norovirus and *Clostridium difficile* coinfections in adult hospitalized patients

**Objective:** Human norovirus (HuNoV) and *Clostridium difficile* are common causes of infectious gastroenteritis in adults in the US. However, limited information is available regarding HuNoV and *C. difficile* coinfections. Our study was designed to evaluate the prevalence of HuNoV and *C. difficile* coinfections among adult patients in a hospital setting and disease symptomatology.

**Study design and setting:** For a cross-sectional analysis, 384 fecal samples were tested for the presence of *C. difficile* toxins from patients (n=290), whom the provider suspected of *C. difficile* infections. Subsequent testing was then performed for HuNoV genogroups I and II. Multinomial logistic regression was performed to determine symptoms more frequently associated with coinfections.

**Results:** The final cohort consisted of the following outcome groups: *C. difficile* (n=196), *C. difficile* + HuNoV coinfection (n=40), HuNoV only (n=12), and neither (n=136). Coinfected patients were more likely to develop nausea, gas, and abdominal pain and were more likely to seek treatment in the winter season compared with individuals not infected or infected with either pathogen alone.

**Conclusion:** Our study revealed that patients with coinfection are more likely to experience certain gastrointestinal symptoms, in particular abdominal pain, suggesting an increased severity of disease symptomatology in coinfected patients.

**Keywords:** human norovirus, *C. difficile*, gastroenteritis, coinfection

**What is new?**
- The key findings are that 1) human norovirus (HuNoV) and *Clostridium difficile* coinfections are relatively frequent, 2) coinfected patients are more likely to develop nausea, gas, and abdominal pain, and 3) they are more likely to reside in a communal setting than those not infected.
- The increased number of follow-up visits associated with HuNoV and *C. difficile* coinfections suggests an increased severity of gastrointestinal disease symptomatology in coinfected patients.
- A greater awareness of the high frequency of HuNoV/*C. difficile* coinfections, particularly in communal settings, may improve patient management and infection control.
Introduction

Acute gastroenteritis (AGE), defined as diarrheal disease of rapid onset potentially accompanied by nausea, vomiting, fever, or abdominal pain, is a major cause of illness in the US resulting in ~179 million episodes of AGE each year. Human norovirus (HuNoV) is the leading cause of AGE outbreaks in the US. In addition, AGE is also an important cause of mortality worldwide, especially in children. In the US, the number of people, mostly elderly, who have suffered a gastroenteritis-related death has doubled during the last decade with Clostridium difficile representing the main and HuNoV the second leading infectious causes.

C. difficile is a Gram-positive, spore-forming, and toxin-producing bacterium. It is the most common cause of health care-associated infectious gastroenteritis in the US, causing ~3 million infections yearly that result in a significant burden of diarrhea and colitis per year. Over the past decade, the incidence and severity of C. difficile infections (CDIs) has been increasing. C. difficile invades the disrupted intestinal microbiota following antibiotic treatment, and pathogenesis is mainly attributed to the two predominant toxins, TcdA and TcdB. Treatment of CDI includes antibiotic discontinuation, administration of specific antibiotics, such as metronidazole or vancomycin, or in severe cases, fecal transplants. However, colonization does not always result in active infection and a proportion of those exposed become asymptomatic carriers.

HuNoVs are nonenveloped, single-stranded, positive-sense ribonucleic acid (RNA) viruses that cause ~20% of all cases of AGE worldwide. In the US alone, these viruses cause ~21 million infections each year. Approximately 5.5 million cases, over half (58%) of all food-borne infections are caused by HuNoVs, which cost an estimated ~US$ 5.8 billion. Noroviruses are divided into at least seven genogroups (G), three of which infect humans (GI, GII, and GIV). Genogroup II genotype 4 (GI.4) noroviruses are the most prevalent and clinically significant HuNoVs, causing approximately two-thirds of all outbreaks. Although, GI.17 strains are emerging and increasing in prevalence. HuNoV symptoms include vomiting and diarrhea, which are rapid in onset but usually resolve in 12–60 hours. Virions are expelled in vomitus and/or feces of symptomatic and asymptomatic carriers for transmission by the fecal-oral route. There are currently no specific treatments approved for HuNoV infections aside from supportive care. Despite their health importance, little is known about the association between C. difficile and HuNoVs in the host. Although coinfections with both pathogens have been reported, the cohorts were very limited in number. Both pathogens have been mistaken as an infectious cause for the other because these pathogens share similar symptoms (primarily diarrhea, nausea, abdominal pain, and fever). One study postulated an antagonistic relationship between the two pathogens, in that the pathogens compete against one another and limit the other’s pathogenicity. However, the risk factors and clinical features associated with coinfections are not well understood. Therefore, the aim of our study was to assess the prevalence of coinfection in a cohort of patients with gastroenteritis, and to examine the symptomatology and characteristics of those coinfected with C. difficile and HuNoV. Findings reported herein may help with the clinical management of patients with gastrointestinal symptoms, as well as infection control and prevention.

Methods

Study population

The study population has been previously described. A cross-sectional study was conducted at the University of Michigan Health System (UMHS, Ann Arbor, MI, USA), following approval by the Institutional Review Board at the University of Michigan. Symptoms were determined from individual retrospective patient data questionnaires and electronic patient records from UMHS at any time prior to diagnosis of CDI and written informed consent was received from all patients from October 2010 to August 2013. All subjects were adults (18 years and older), who had been admitted for treatment at UMHS and the provider suspected CDI. CDI testing is ordered for symptomatic patients; for example, those with antibiotic-associated diarrhea. For the purpose of this analysis, individuals who were discharged and sought treatment again at least 2 weeks after the initial treatment and hospital discharge were considered reinfected and analyzed separately. For individuals with multiple C. difficile test samples, the first test result and sample was used in this study. Controls were patients who tested negative for CDI.

Stool collection and handling

Stools samples from hospitalized inpatients and ambulatory outpatients presenting to the main hospital or UMHS off-site facilities were obtained, stored in Cary-Blair transport media and tested for C. difficile as described. Specifically, clinician-ordered stool specimens were tested for the presence of C. difficile glutamate dehydrogenase antigen and toxins A and B using the C. DIFFICILE QUIK CHEK COMPLETE test (Techlab, Inc., Blacksburg, VA, USA). All antigen/toxin
Samples were first analyzed by real-time PCR and RT-nested several years of storage and at least two freeze/thaw cycles. Controls, respectively. Sterile water were used in each run as positive and negative turer’s recommendations. A HuNoV-positive stool sample and StrataClone PCR cloning system according to the manufacturer’s recommendations. The MD145-12 (nt 5007 to 5099, accession number AY032605, amplifying 93 nt from the ORF1/2 junction of GII.4 HuNoV were previously described. Two-tailed P-value of <0.05 was considered to be significant. The patient characteristics of age, sex, race, weight, residence when sampled, and season of diagnosis as well as number of follow-up visits, use of a feeding tube, antibiotic usage within the past 7 days, and history of chronic conditions (defined as history of either cardiac disease, lung disease, kidney disease, diabetes, autoimmune disease, inflammatory bowel disease, and/or acquired immunodeficiency syndrome/human immunodeficiency syndrome). Symptomatic factors included vomiting, nausea, abdominal pain, urgency, and gas.

Data analysis
For statistical analysis, stool samples were categorized as coinfection, HuNoV only, C. difficile only, or neither based on laboratory results. A data set of 384 stool samples from 290 patients was used for unadjusted and adjusted analysis. Statistical analyses were performed using SAS/STAT software, Version 9.3 of the SAS System for Windows (SAS Institute Inc., Cary, NC, USA) and Stata MP 14.0 (StataCorp LP, College Station, TX, USA). A two-tailed P-value of <0.05 was considered to be significant. The patient characteristics of age, sex, race, weight, residence, and previous conditions were noted at the time of study entry. Pearson’s chi-squared test was used for categorical variables and one-way analysis of variance for continuous variables. For antibiotic use in the past 7 days, use of feeding tube, and season (December

Norovirus and C. difficile coinfections

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Determining a detection cutoff for HuNoV-positive samples
Following quantitative PCR for HuNoV, all cycle threshold (CT) values were graphed. Similar to the study by Trang et al, we observed a biphasic distribution (data not shown). CT values in one group ranged from 25 to 34, while those in the second group ranged from 35 to 38. To decrease the potential of false positives, a CT value of 34 was chosen as a cutoff for HuNoV detection. Thus in this study, samples with CT values ≤34 were considered HuNoV positive, while samples with a CT value >34 were considered HuNoV negative.

Clinical epidemiology
Symptomatology, epidemiologic, and laboratory factors were analyzed to determine if certain variables were predictive of coinfection in comparison to infection with one pathogen. Epidemiologic factors included patient age, sex, race, weight, residence when sampled, and season of diagnosis as well as number of follow-up visits, use of a feeding tube, antibiotic usage within the past 7 days, and history of chronic conditions (defined as history of either cardiac disease, lung disease, kidney disease, diabetes, autoimmune disease, inflammatory bowel disease, and/or acquired immunodeficiency syndrome/human immunodeficiency syndrome). Symptomatic factors included vomiting, nausea, abdominal pain, urgency, and gas.

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HuNoV testing
To test for the presence of HuNoV, viral RNA was extracted from stool samples using the QIAamp Viral RNA Mini Kit (Qiagen, Redwood City, CA, USA) according to the manufacturer’s protocol. RNA was further cleaned up with the TURBO DNA-free Ambion RNA kit (Thermo Fisher Scientific, Waltham, MA, USA) following the manufacturer’s recommendations. Samples were first screened for GII HuNoV by TaqMan quantitative reverse transcription-PCR using CFX96 Real Time System (Thermo Fisher Scientific). Oligonucleotide primers and probe specific for the junction between ORF1 and ORF2 of the HuNoV genome were previously described. A 20 µL PCR reaction was performed using iScript one-step quantitative reverse transcription-PCR kit (Bio-Rad Laboratories Inc., Hercules, CA, USA) with the following conditions: 50°C (10 minutes), 95°C (5 minutes); 40 cycles at 94°C (10 seconds) and at 60°C (30 seconds). Standard curves were generated from ten-fold dilutions of plasmid pSC-GII in each run. The pSC-GII plasmid was constructed by pSC-A-amp/kan using the TURBO DNA-free Ambion RNA kit (Thermo Fisher Scientific, Waltham, MA, USA) following the manufacturer’s recommendations.

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Results

HuNoV screening of study cohort

To determine the level of coinfection between *C. difficile* and HuNoV, we screened 384 stool samples from 290 UMHS patients for the presence of HuNoV. The age (mean ± standard deviation) of all patients at the time of study entry was 56.2 years (±16.4 years) and 44% were male. Of the 384 stool samples collected, 236 samples were positive for *C. difficile*, while 148 samples tested negative for *C. difficile*. Of the positive *C. difficile* samples, 40 also tested positive for HuNoV (17%). Of the 148 negative *C. difficile* samples, 12 tested positive for HuNoV (8%). A total of ten HuNoV samples were successfully sequenced. Genotyping of nine samples indicated GII.P4/GII.4 2009 HuNoV strains, while one sample contained GII.Pg/GII.1 HuNoV. Fecal samples from 125 patients tested negative for either pathogen and were used as controls. Taken together, the final cohort had the following outcome groups: *C. difficile* only (n=196 specimens, 131 patients), *C. difficile* + HuNoV (n=40 specimens, 24 patients), HuNoV only (n=12 specimens, ten patients), and neither (n=136 specimens, 125 patients).

Age, sex, race, and weight were similar across the four outcome groups at the time of enrollment (Table 1). However, there was a difference in infection depending upon where the patient resided (P=0.0004). For those with a coinfection, 21% lived in a rehabilitation center, nursing home, assisted living center, or dormitory, while only 3% of the controls lived in such residences. When included in a multinomial logit model (accounting for clustering), the difference between coinfection (specifically) and controls was significant for residential living (P<0.001). There was also a significant difference in residences for those with *C. difficile* and controls (P=0.014); 11% of those with *C. difficile* resided in communal residences compared with 3% of the controls. In addition, no significant difference was observed in infections in the winter season (P=0.090) across all groups. However, a direct comparison of coinfection versus control (47% of coinfections occurred in the winter compared with 26% for controls) within a multinomial logit model demonstrated significance (P=0.017).

Analysis of recent antibiotic use indicated no difference among outcomes, while no differences were observed for the symptoms of gastrointestinal urgency (P=0.920) or gas (P=0.013) (Table 1). Nausea and vomiting were particularly present in patients with *C. difficile* (75%) (Figure 1). Vomiting symptoms were typically combined with nausea and few to no patients presented with vomiting only (Figure 1). Furthermore, abdominal pain was the symptom that best differentiated those patients with a coinfection as abdominal pain occurred in 70% of those with a coinfection compared with 57% in controls (P=0.002) (Table 2). No significant difference in abdominal pain was observed between patients with *C. difficile*-only infections and hospitalized controls.

To identify clinical symptoms and characteristics that differentiated coinfected patients from the other outcome groups, a multinomial logistic regression model was used to calculate odds ratios (ORs) and P-values for the association between symptoms and infection outcomes (ie, coinfection, norovirus only, *C. difficile* only, or neither infection) with adjustment for patient characteristics. Neither infection was used as the reference category. All ORs were adjusted for age, sex, and repeated sample collection after 2 weeks.

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Coinfection</th>
<th>Norovirus</th>
<th><em>Clostridium difficile</em></th>
<th>Neither</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, mean (SD)</td>
<td>60.2 (16.1)</td>
<td>58.4 (11.8)</td>
<td>54.8 (18.3)</td>
<td>56.7 (14.6)</td>
</tr>
<tr>
<td>Female, n (%)</td>
<td>14 (58)</td>
<td>5 (50)</td>
<td>68 (52)</td>
<td>74 (59)</td>
</tr>
<tr>
<td>Race (White), n (%)</td>
<td>22 (96)</td>
<td>7 (78)</td>
<td>114 (91)</td>
<td>110 (91)</td>
</tr>
<tr>
<td>Weight (lb), mean (SD)</td>
<td>149 (50)</td>
<td>174 (68)</td>
<td>164 (53)</td>
<td>168 (65)</td>
</tr>
<tr>
<td>Residence, n (%)</td>
<td>181 (55)</td>
<td>226 (27)</td>
<td>183 (54)</td>
<td>199 (53)</td>
</tr>
</tbody>
</table>

Notes: 1Rehabilitation center (n=10), assisted living center (n=9), nursing home (n=3), dormitory (n=1), other (n=1). 2Heart disease, lung disease, kidney disease, diabetes, autoimmune disease, inflammatory bowel disease, and/or AIDS/HIV.

Table 1 Characteristics of patients by infection status
groups with adjustment for demographic characteristics, repeated sampling (after 2 weeks post original sample), and using a quantitative PCR CT cutoff of 34 for HuNoV, there was a significant interaction between nausea and vomiting and infection status (Table 3). Coinfection was more likely in patients who presented with abdominal pain (OR = 4.77, 95% confidence interval [CI]: 2.09–10.9], nausea (OR = 4.30, 95% CI: 1.65–11.3), gas (OR = 2.30, 95% CI: 1.03–5.10), or had a diagnosis during the winter season (December–February) (OR = 2.30, 95% CI: 1.0–4.82). Patients with C. difficile-only infection also had significant nausea and abdominal pain (OR = 1.94, 95% CI: 1.04–3.60 and OR = 1.70, 95% CI: 1.06–2.73, respectively). The ORs were greater in coinfected patients compared with C. difficile-only infection suggesting a greater prevalence of nausea and abdominal pain in the coinfected group. When coinfection was directly compared with C. difficile only, abdominal pain remained more prevalent in those with coinfection (OR = 2.10, 95% CI: 1.11–3.98, P = 0.023). In addition, follow-up visits were greater in coinfection versus C. difficile only (P = 0.042). Those with HuNoV-only infections were significantly more likely to experience vomiting (OR = 5.47, 95% CI: 0.85–34.8) compared with coinfected and C. difficile-only infected patients, who showed no significant association for vomiting as a symptom (OR = 1.02, 95% CI: 0.40–2.63 and OR = 1.21, 95% CI: 0.63–2.35, respectively). As expected, patients with C. difficile-only infection showed a 2.89 greater odds (95% CI: 1.47–5.69) of having used antibiotics in the 7 days prior to sampling (P = 0.002). Taken together, these data indicate that coinfected patients were more likely to develop nausea, gas, and abdominal pain, and were more likely to seek treatment in the winter season compared with those infected with either pathogen alone.

Table 3 Adjusted ORs for disease outcome using multinomial logistic regression

<table>
<thead>
<tr>
<th>Disease outcome</th>
<th>Coinfection</th>
<th>Norovirus only</th>
<th>C. difficile only</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vomiting</td>
<td>0.02</td>
<td>0.40–2.63</td>
<td>0.67</td>
</tr>
<tr>
<td>Nausea</td>
<td>4.53</td>
<td>0.10–11.3</td>
<td>0.003</td>
</tr>
<tr>
<td>Vomiting/nausea</td>
<td>0.008</td>
<td>&lt;0.001</td>
<td>0.001</td>
</tr>
<tr>
<td>Abdominal pain</td>
<td>2.47</td>
<td>2.09–10.9</td>
<td>0.001</td>
</tr>
<tr>
<td>Urgency</td>
<td>1.53</td>
<td>0.0–1.83</td>
<td>0.02</td>
</tr>
<tr>
<td>Gas</td>
<td>2.30</td>
<td>1.03–5.01</td>
<td>0.04</td>
</tr>
<tr>
<td>Winter season diagnosis</td>
<td>2.30</td>
<td>1.00–4.82</td>
<td>0.03</td>
</tr>
<tr>
<td>Chronic condition history</td>
<td>1.34</td>
<td>0.41–4.35</td>
<td>0.62</td>
</tr>
<tr>
<td>Follow-up visit number</td>
<td>1.71</td>
<td>0.78–3.73</td>
<td>0.18</td>
</tr>
<tr>
<td>Use of feeding tube</td>
<td>1.08</td>
<td>0.46–2.57</td>
<td>0.86</td>
</tr>
<tr>
<td>Antibiotic use in past 7 days</td>
<td>2.07</td>
<td>0.70–6.16</td>
<td>0.19</td>
</tr>
</tbody>
</table>

Notes: *Individuals without norovirus or C. difficile infection constituted the reference category. All ORs were adjusted for age, sex, and repeated sample collection after 2 weeks. *The interaction term for nausea and vomiting, A, P-value of <0.05 was considered significant. Abbreviations: CI, confidence interval; OR, odds ratio.
Discusson

The aim of this study was to determine the prevalence of *C. difficile* and HuNoV coinfection in order to aid in clinical diagnosis, patient management, and infection control in the future. Our comprehensive analysis of coinfection with HuNoV and *C. difficile* in adult hospitalized patients indicated that coinfections with these two pathogens occur relatively frequently in this patient population. Our findings further indicate that patients with CDI experiencing nausea with or without vomiting, abdominal pain, and/or gas have a greater likelihood of being coinfected with HuNoV, especially during the winter season.

Several main conclusions can be drawn from the data presented in this study. First, our study confirmed findings from previous studies regarding the occurrence of coinfections of *C. difficile* and HuNoV. Previous studies in a pediatric population reported coinfection rates that ranged from 13% to 17%,16,18 while 13% coinfection rates were reported in hospital-acquired CDI,40 and 16% in a long-term care facility.17 Using a next-generation sequencing approach, an 18% coinfection rate (4/22) was detected.31 The prevalence of coinfection in our cohort of 384 samples was 10%. These slightly higher rates in previous studies may be due to a variety of reasons, such as smaller sample sizes, children being more likely to have asymptomatic *C. difficile* colonization in the intestinal tract, increased person-to-person transmission within long-term care facilities due to close proximity, or because we included a threshold for HuNoV positivity in our study.

The overall finding of coinfection raises the question regarding changes in disease severity in *C. difficile-* or HuNoV-infected patients compared with those coinfected with both pathogens. The second finding from our study was that patients with coinfection showed the highest mean number of follow-up visits compared with the other groups, consistent with the idea that concurrent pathogen infection may cause more severe disease outcomes than infection by either pathogen alone. Therefore, data presented herein indicate that the two pathogens may work synergistically when infecting concurrently to yield an intensified clinical impact in the host.

Third, our data demonstrated that the symptomatology of coinfections is not necessarily a combination of symptoms experienced with infection by a single pathogen. Vomiting is a well-characterized symptom during HuNoV infection.32 However, vomiting is not a feature of CDI and no association with vomiting was seen in *C. difficile*-infected patients in our study. Interestingly, no association between vomiting and coinfection was observed. Instead, coinfection of *C. difficile* and HuNoV was associated with nausea, abdominal pain, and gas, symptoms more frequently experienced by patients infected with *C. difficile*. These data indicate that symptoms caused by CDI may be dominant over HuNoV-induced symptoms in the setting of coinfection. Thus, in clinical settings, it may be beneficial (eg, for infection control or patient management) to take into account that patients who are diagnosed with CDI and present the symptoms of abdominal pain, gas, and nausea with or without vomiting may also be coinfected with HuNoV. This would be particularly important during the winter season because our findings also suggest that the season of diagnosis can impact the likelihood of coinfection in patients as the incidence of coinfection was significantly higher during the winter months. HuNoV infections often peak in the winter months in the Northern Hemisphere.33,34 In contrast, CDI does not show seasonality trends because CDI rates are largely a result of antibiotics use.4 Therefore, the overall greater prevalence of HuNoV in the population during the winter months likely resulted in the greater odds of coinfection during winter that we observed in this study.

The current study also has certain limitations. One was the use of storage buffer for fecal samples. Transport of stool samples containing enteric bacterial pathogens often uses Cary Blair media (pH >8),35 and it was used in our study. However, HuNoVs are inactivated by alkaline pH.36 At pH 8, increased solvent exposure of tyrosine residues and secondary structure changes were observed, resulting in reversible dissociation of the viral capsid. Thus, the pH of the Cary Blair buffer was not optimal for viral transport/storage and may have resulted in degradation of viral particles over time and a loss of HuNoV titers in the sample. This most likely explains our low sequencing success rate since samples were sequenced several years after collection and repeated freeze/thaw events. Therefore, it is also unlikely that the HuNoV genome titers measured reflect accurately the viral titers at the time of collection. Furthermore, the inadequate storage buffer may have resulted in an underreporting of HuNoV infections, particularly since we utilized a threshold for HuNoV titers to prevent false-positive classification. Therefore, for any future studies of viral and bacterial coinfection, fresh, unmodified samples should be collected. Alternatively, parallel stool aliquots may be collected in viral as well as bacterial transport media.

Though our study design has limitations, the results may provide important guidelines for clinical practice and infection prevention. The identification of HuNoV in patients originally diagnosed to only have CDI is crucial.
in the clinical setting due to the highly contagious nature of the virus, where as little as 18 HuNoV particles were estimated to be sufficient to cause an infection. Additionally, HuNoV shedding for weeks to months after the resolution of symptoms makes these viruses a key pathogen to be regulated by infection control in acute care settings. In addition, while limited studies have identified HuNoV/CDI coinfection in hospitalized and long-term care facility patients, our study serves as the first comprehensive analysis of coinfection with these two pathogens in adult hospitalized patients. This increased knowledge about the potential burden of coinfection in patients with gastrointestinal symptoms in clinical settings may result in improved diagnostic procedures.

In summary, we confirmed previous studies that coinfections of HuNoV and C. difficile occur in adult patients presenting clinical symptoms of gastroenteritis. Our study revealed that patients with coinfection are more likely to experience certain gastrointestinal symptoms – abdominal pain, gas, and nausea with or without vomiting – and show increased numbers of follow-up visits, suggesting direct or indirect interactions between these pathogens in the host during infection. These findings are consistent with an increased severity of disease in coinfected individuals but future studies are needed to identify the underlying mechanisms of pathogen interactions within the host, or whether the order of pathogen acquisition dictates patient outcomes. Regardless, clinicians diagnosing patients with CDI should be aware of possible HuNoV coinfections and the consequences for clinical treatment and infection prevention management.

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Disclosure
The authors report no conflicts of interest in this work.

References