Clinical and Experimental Health Sciences

Prevalence of Norovirus Coinfection in *Clostridioides difficile* Toxin Positive Patients

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ABSTRACT

Objective: In this study we aimed to evaluate the prevalence of norovirus genogroups I and II and *C.difficile* coinfection among patients with gastroenteritis symptoms.

Method: A total of 76 patients with diarrhea were included in the study. Of these, 40 are children (<18y), 23 are adults between the ages of 18-65y, and 13 are patients older than 65 years. All these were *C.difficile* toxin GDH/Toxin A+B (BIOTEC, Spain) positive. In these toxin positive stool samples, Norovirus GI and GII antigen was studied by 2 methods; i.ELISA (R Biopharm, Darmstadt, Germany) and ii.polymerase chain reaction (RT – PCR). We compared the results of the antigen test (ELISA) with those of PCR for the detection of norovirus in stool specimens. SPSS 19.0 statistical program was used to evaluate the data of the research.

Results: Out of 76 stool samples tested, 3 (3.9%) were positive for norovirus by ELISA. Subsequent RT-PCR identified norovirus GI and GII in 7 samples (9.2%). Concerning RT-PCR, the sensitivity of the ELISA test was 42.8%, and the specificity was found as 96%.

Conclusion: The study identified a 9.2% rate of co-infection with *C. difficile* and norovirus, with this co-infection being particularly prevalent in children. This finding emphasizes the critical need to consider both co-infection and *C. difficile* infection as potential causes of diarrhea in hospitalized patients, especially those under 18 or over 65 years old.
Keywords: *C. difficile*, Coinfection, ELISA, Norovirus, PCR

1. INTRODUCTION

C. *difficile* is a Gram-positive, spore forming and toxin producing anaerobic bacteria. It is the most common cause of health care-associated infectious gastroenteritis and can cause a wide range of infections, from asymptomatic intestinal colonization to severe diarrhea, pseudomembranous colitis, and toxic megacolon. In recent years, a significant increase in morbidity and mortality due to *C. difficile* infections has been observed. CDC has placed this microorganism in a priority group for the prevention of health-related infections (1). The most important risk factors for the development of community-acquired *C. difficile* infection include being 65 years of age or older, while healthcare-associated *C. difficile* infection is also associated with hospitalization or nursing home residence. Immunosuppressive conditions such as hematologic malignancy. Although *C. difficile* infections are mostly healthcare-associated infections, they are increasingly occurring in the community among people without classic risk factors. Recently, some studies have shown that more than 35% of patients with community-acquired (CA) *C. difficile* infection (CDI) do not use antibiotics, and more than 50% of these patients report symptoms such as nausea or vomiting that are not present in classic symptoms of CDI (2).

This suggests that some symptoms may occur due to co-infections with other pathogens, especially viruses. Noroviruses are viruses that are common causes of acute gastroenteritis globally (3). Noroviruses are nonenveloped, single-stranded, positive – sense ribonucleic acid (RNA) viruses. The genogroups that infect humans are GI (8 genotypes), and GII are the most common cause of NoV outbreaks worldwide (4,5,6).

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Despite both *C. difficile* and NoVs posing major health threats, their potential interaction within the host is largely unexplored. By disrupting the intestinal microbiota or natural host defenses, viruses can create favorable environmental conditions for the colonization of *C. difficile* and thus cause co-infections (7,12). Although coinfections with both pathogens have been reported, the number of studies are limited in number.

Therefore, in this study, we aimed to detect the prevalence of norovirus co-infection in *C. difficile* toxin-positive patients with gastroenteritis using PCR and ELISA methods.

2.METHODS

2.1. Patients and Specimen Collection

Stool samples sent to Clinical Microbiology Laboratory between January 2019 and March 2020 from patients with gastroenteritis symptoms and were positive for *C. difficile* toxin were included in the study. Samples were examined macroscopically and microscopically. Direct examination results were recorded (13).

2.2. Detection of C.difficile Toxin

The stool samples were tested for the presence of *C. difficile* glutamate dehydrogenase antigen and toxins A and B using chromatographic immunoassay (GDH/Toxin A+B combo card test, Certest, Spain). Amorphous stool samples, which were negative with this method but inflammatory cells were present in direct microscopy, were directly inoculated in cycloserine, cefoxitin agar (CLO agar; BioMerieux, France) for *C. difficile.* Based on the colony's appearance, size, color, and other characteristics, the bacteria were identified by MALDI-TOF MS (VITEK MS®, bioMerieux, France). Colonies were inoculated into brain-heart infusion broth (BHIB; Becton Dickinson, Germany) and incubated in an anaerobic environment. After five days of incubation, the liquid medium was centrifuged at 3000 rpm for 20 minutes and the supernatant was removed and the presence of toxin was investigated by chromatographic immunoassay (GDH/Toxin A+B combo card test, Certest, Spain). Remaining stool samples were aliquoted and stored at −80°C.

2.3. Detection of Norovirus Antigen by ELISA Method

To detect norovirus antigen, an ELISA kit (RIDASCREEN, third generation, R-Biopharm, Darmstadt, Germany), which can determine GI and GII genotypes, was used. Stool samples stored at – 80°C were thawed at room temperature for 30 minutes, diluted 10% with the ELISA kit buffer, and centrifuged before following the manufacturer's protocol. The assay validity was confirmed by negative control OD < 0.2 and positive control OD > 0.5. Samples were considered positive if their OD values exceeded the calculated cut-off (negative control OD + 0.15) by 10% or more, otherwise negative. Kit documentation claims no cross-reactivity, 80% sensitivity, and 100% specificity (13).

2.4. Detection of Norovirus in Stool with RT-PCR Method

RIDA®GENE Norovirus I & II real-time PCR (R-Biopharm, Darmstadt, Germany), a real-time in vitro diagnostic kit, was used for the detection of norovirus by PCR in stool. For RNA extraction from feces, the HigherPurity™ Viral RNA Extraction Kit (Canvax, Mexico) was used according to the manufacturer's instructions. PCR was performed with Rotor-Gene 6000 Real-Time PCR (Corbett Research, Qiagen GmbH).

PCR procedures were carried out as follows: reverse transcription for 10 minutes at 58°C, initial denaturation for 1 minute at 95°C, denaturation for 15 seconds at 95°C and primer binding/extension for 30 seconds at 55°C, for a total of 45 cycles.

2.5. Clinical Epidemiology

In this study, we analyzed the epidemiologic factors to determine whether certain parameters were predictive of coinfection. Epidemiologic factors included age, sex, clinics (inpatient,outpatient), symptoms (diarrhea, nausea, vomiting, fever) history of the patient, antibiotic usage, history of chronic conditions (inflammatory bowel disease, malignancy, diabetes, autoimmune disease), antibiotic usage, laboratory findings (stool culture for *Salmonella* spp., *Shigella* spp., *Campylobacter* spp. , Rotavirus Ag, Adenovirus Ag positive with Rota-Adeno Card Test (CerTest, Biotec, Spain))

2.6. Data Analysis

SPSS 19.0 statistical program was used to evaluate the data of the research. Data were analyzed using frequency and percentage, descriptive statistical analysis. When comparing variables of categorical data such as clinical and demographic, the chi-square test was used. Statistical significance level $p \leq 0.05$ was determined.

Ethical Approval

The study was approved by Marmara University Ethics Committee, Noninvasive Clinic Ethics Committee (Approval date-no. 14.01.2019-13).

3. RESULTS

The study included a total of 76 patients 39 (51.3%) were males and 37 (48.6%) were females. Of these, 40 are children (<18), 23 are adults between the ages of 18-65, and 13 are patients older than 65. Twenty-one of the children are between the ages of 2-3 In microscopic examination, leukocytes were seen in 20 (26.3%) of the samples, while leukocytes and erythrocytes were seen in 13 (17.1%). Of the 76 *C. difficile*-positive samples, 70 were toxin-positive directly from the sample, while 6 were positive from toxigenic culture. No association was found between the detection of toxin positivity, either directly from the sample or through toxigenic culture, and norovirus positivity. Norovirus antigen was detected in 9.2% (n:7) of the samples by PCR, whereas the ratio was 3.9% (n:3) by ELISA. As a result, 3 samples were detected positive by both RT-PCR and ELISA, while 4 samples were only positive by RT-PCR. Using RT-PCR as a reference, the sensitivity of the ELISA test was found to be 42.8% and the specificity was 94.7% (Table 1). In our study, when adenovirus and rotavirus positivity was included, the viral co-infection rate was found to be 19.7% (n:15). Except for 7 patients with norovirus coinfection, rotavirus antigen was detected in 5 patients (6.5%) and adenovirus antigen in 3 patients (3.9%).

For bacterial coinfection, *Salmonella enterica* was the only isolated bacterial pathogen among the samples**.** Norovirus coinfection increased the likelihood of experiencing symptoms like nausea, vomiting, and fever, with a prevalence of 42.7% in coinfected patients compared to just 27.8% in non-coinfected patients. In the clinical history of the patients 18 patients were diagnosed with Inflammatory Bowel Disease, 17 patients were diagnosed with malignancy and 4 patients were followed up due to Cystic Fibrosis. 50% of the patients had an underlying condition (Table 2). Among prior medication exposures, antibiotherapy rate was very similar in each group. No statistically significant difference was observed in demographic characteristics such as age groups, gender and clinical findings of co-infected patients (p>0.05 for all groups).

Table 1. *Results of Norovirus antigen detected by RT-PCR and ELISA according to age groups*

Age	METHODS			
	RT PCR		ELISA	
	positive	negative	positive	negative
2-18	5	35	2	38
18-65	1	22	0	23
>65	1	12	1	12
TOTAL	7	69	3	73

Table 2. *Comparison of demographics, prior healthcare and medication exposures, and clinical characteristics between co-infected and non-co-infected patients*

Characteristics	Norovirus Co-infected cases (n/%) (7/9.2)	Norovirus Non-co-infected cases (n/%) (69/90.7)
Age group		
2-3	**0(0)**	**21(30.4)**
3-18	**5(71.4)**	**14((20.2)**
18-65	**1(14.2)**	**22(31.8)**
65 and over	**1(14.2)**	**12(17.3)**
Male/ Female	**3(42.8)/ 4(57.1)**	**41(59.4)/ 30(43.4)**
Inpatient/ Outpatient	**4(57.1)/ 3(42.8)**	**35(50.7)/ 36(52.1)**
Diarrhea	**7(100)**	**69(100)**
Nausea or vomiting	**1(14.2)**	**9(13.04)**
Fever	**2(28.5)**	**10(%14.4)**
Patient history		
Inflammatory bowel disease	**1(14.2)**	**17(24.6)**
Malignancy	**2(28.5)**	**15(21.7)**
Cystic fibrosis	**0(0)**	**4(5.7)**
Diabetes mellitus	**0(0)**	**4(5.7)**
Surgical procedure	**1(14.2)**	**5(7.2)**
Prior medication exposures		
Any antibiotics	**4(57.1)**	**40(57.9)**
Charlson comorbidity index ≥1	**6(85.7)**	**53(76.8)**
Laboratory findings		
Stool culture positive**	**1**	
Rotavirus Ag positive	**0(0)**	**5(7.2)**
Adenovirus Ag positive	**0(0)**	**3(4.3)**
Immunosuppressants*	**3(42.8)**	**29(42)**

**: Patients with a history of immunosuppressive medication or receiving chemotherapy. ** Salmonella enterica was reported for the stool culture*

4. DISCUSSION

In this study we aimed to determine the prevalence of *C. difficile*-norovirus coinfection in order to aid the clinical diagnosis and treatment for patient management. The pathogenesis of *C. difficile*-norovirus coinfection is not fully understood, but it is suggested that C*. difficile* toxins may alter intestinal homeostasis, predisposing to viral coinfections. Norovirus is one of the most leading causes of gastroenteritis in sporadic cases or epidemics and frequently studied virus in *C. difficile* coinfections (12,14,15). The prevalence of norovirus coinfection was detected as 9.2% in our study. Previous studies in a pediatric population reported coinfection rate as 12% and in adults this ratio ranged from 8.9% to 10% (16,17,18).

In a meta-analysis, 31 different studies conducted on children under the age of 18 suffering from diarrhea were examined. Of the total 10,201 patients, 16.1% were *C. difficile* positive and 10% of them were reported as norovirus co-infected (19). The literature shows that norovirus co-infection rates can reach higher levels during outbreak situations. In a study examining the Norovirus outbreak in Germany between 2002 and 2012, the results of 44 outbreaks in 5 different German hospitals were reported, and at least one of 9 outbreaks (20%) was reported to have *C. difficile* and norovirus co-infection (20). During our study period there was no evidence of norovirus outbreak. When we evaluated the laboratory epidemiological data, we found out that rotavirus antigen was also detected in 5 patients (6.5%) and adenovirus antigen in 3 patients (3.9%), in addition to the 7 patients with norovirus co-infection. Overall viral co-infection rate was found to be 19.7%. When frequently detected viral co-infection agents are evaluated, it is seen that norovirus incidence is followed by adenovirus and rotavirus, in line with world data (17). Since the detection of *C. difficile* under the age of two is generally considered colonization, this age group was not included in our study (21). Five of the patients with norovirus co-infection are in the 3-18 age group and 1 is over 65 years old. In our study, *C. difficile*-norovirus co-infection was detected in 12.5% (n:5) of those under 18 years of age. The rate we found appears to be above the literature values for this age group. In our study, the norovirus co-infection rate was found to be 7.6% (n:1) in adult patients over 65 years of age. The patient in this group had a history of multiple myeloma, and a sample was taken due to diarrhea that developed after clarithromycin and piperacillin/tazobactam treatment for pneumonia. Upon detection of coinfection, the patient was started on metronidazole and vancomycin treatment. It is known that the only patient between the ages of 18-65 had a history of meropenem treatment due to ventriculitis and was given metronidazole for the treatment of diarrhea.

C. difficile norovirus coinfection is more common in immunosuppressed patient groups. In a study conducted in China, *C. difficile* was reported in 68% (n:55) of 81 individuals living with HIV who complained of diarrhea, while *C. difficile*-norovirus co-infection was reported in 16.3% (9/55) of them (22). No HIV positive patients were included in our

study. However, 38.1% (n:29) of the patients were patients receiving immunosuppressive therapy. It is noteworthy that more coinfections with both norovirus and other viral agents (rotavirus, adenovirus) were detected in these patient groups.

When all patients included in the study were evaluated, it was observed that 65% (n:26) of the patients under the age of 18 had a history of previous antibiotic use and 42.5% were treated with metronidazole. It was observed that 47.2% of adult patients (n:17) had a history of antibiotic use, and 45.8% of them used metronidazole for the treatment of *C. difficile*. Four of the patients with norovirus co-infection had a history of previous antibiotic use (vancomycin, meropenem, clarithromycin and piperacillin/tazobactam), and all of these patients were treated by metronidazole.

As a result, when investigating the etiology of diarrhea in hospitalized patients, especially in patients under the age of 18 and over the age of 65, the presence of co-infection, as well as *C. difficile* infection, should be taken into consideration.

Another important outcome of our study; is the evaluation of the performance of ELISA and PCR, for the detection of Norovirus. In our study; the sensitivity of the ELISA test was 42.8% and the specificity was 96%, when RT-PCR was taken as the gold standard test. In a study, comparing the performance of ELISA and RT-PCR, the sensitivity of the ELISA test was reported as 66% and 86%, and the specificity was reported as 92.5% and 100%, respectively. Our results are compatible with the literature and suggest that PCR is more suitable for Norovirus.

In conclusion, in addition to *C. difficile* infection, the presence of co-infection should be considered when investigating the etiology of diarrhea in hospitalized patients, especially those under 18 and over 65 years of age. In recent years, panel tests have become increasingly important in the diagnostic approach to gastroenteritis. However, these tests are expensive and not cost-effective for every patient. Although the ELISA tests we used in our study are relatively cheaper tests, there were differences in sensitivity and specificity between them and molecular tests. This suggests that molecular methods, which are the gold standard for detecting norovirus, should also be used routinely outside of outbreak situations. There is still a need for cheaper, faster, and more accurate tests for the detection of co-infections.

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Author Contributions:

Research idea: HŞŞ, Aİ
Design of the study: HŞŞ, Aİ
Acquisition of data for the study: HŞŞ, Aİ
Analysis of data for the study: HŞŞ, FMA, ES, Aİ
Interpretation of data for the study: HŞŞ, FMA, ES, Aİ
Drafting the manuscript: FMA, ES, Aİ
Revising it critically for important intellectual content: FMA, ES, Aİ
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