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Clostridioides difficile (including epidemiology)

Coexistence of *Clostridioides difficile* and *Staphylococcus aureus* in gut of Iranian outpatients with underlying inflammatory bowel disease

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ABSTRACT

Clostridioides difficile and *Staphylococcus aureus* are two well-known pathogens both causing hospital- and community-acquired infections. However, their intestinal coexistence was not well investigated in inflammatory bowel disease (IBD). Herein, we explored the prevalence of *C. difficile*, *S. aureus* and their coexistence in the gut of Iranian patients with IBD. Fecal and colon specimens were obtained from 70 outpatients with underlying IBD, and investigated for the presence of *C. difficile* and *S. aureus*. *C. difficile* isolates were characterised by CE-ribotyping. PCR was used for detection of toxin-encoding genes of *C. difficile* and *S. aureus* isolates. The antimicrobial susceptibility testing of *C. difficile* and *S. aureus* isolates were examined by agar dilution and Kirby-Bauer disk diffusion methods, respectively. Totally, *C. difficile* and *S. aureus* were detected in only 5.7% and 15.8% of IBD flares. Coexistence of *C. difficile* and *S. aureus* was detected in 5.7% of IBD flares. Two different *C. difficile* ribotypes including RT 126 and RT 017 were identified showing toxin profiles of $tcdA^+B^+/cdtA^+B^+$ and $tcdA^+B^+$, respectively. In *S. aureus* isolates, only positivity for the presence of *sea* enterotoxin was detected. *C. difficile* isolates were susceptible to metronidazole, ceftazidime and fidaxomicin. The highest resistance of *S. aureus* isolates was observed against penicillin (92.3%), following amoxicillin-clavulanate (38.5%) and amikacin (30.8%). Our findings demonstrated that patients with IBD flare are more sensitive to acquire coinfection of *C. difficile* and *S. aureus* than remission. However, more robust data is required to study the crosstalk between these enteric infections and their clinical relevance in patients with IBD flare.

1. Introduction

Clostridioides difficile (formerly Clostridium difficile) is one of the major concerns in healthcare associated environments. This Gram-positive spore-forming anaerobe is the leading cause of a range of colonic diseases, eg. antibiotic-associated diarrhea (AAD), pseudomembranous colitis (PMC) and toxic megacolon which can lead to colonic perforation and death of the patient [1,2]. The incidence and severity of *C. difficile* infection (CDI) has been increased during the past two decades, and about 20–30% of patients with AAD experienced laboratory-confirmed CDI [3].

Inflammatory bowel disease (IBD) is a relapsing-remitting disorder consisting of ulcerative colitis (UC) and Crohn's disease (CD), the two most common conditions characterised by chronic relapsing inflammatory states [4]. The main features of CD and UC are defined as abdominal pain, diarrhea and bleeding followed by extraintestinal manifestations in some cases [5,6]. According to the

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different disease activity indices, the disease is classified into two phases named remission and flare [7]. Although the pathogenesis of IBD is not precisely understood, previous studies confirmed the crucial role of intestinal microbiota on the onset of this autoimmune disease [8]. Antibiotic therapies can alter the normal composition of gut microbiota, which in turn may favor colonization of various pathobionts in the mucosal sites of intestinal lumen [9].

Several studies have highlighted the individual role of specific bacterial pathogens in exacerbation of IBD [10–12]. In recent years, CDI was shown to be associated with excess morbidity and mortality along with the elevated risk of hospitalization, increased costs and escalation of therapy in IBD patients [13]. It has been reported that up to 20% of the patients with IBD flares are infected by *C. difficile* [14]. Moreover, colonic involvement, biologic therapies and use of antibiotics reported being as the main risk factors associated with development of CDI among IBDs [15].

The majority of commensal microorganisms, collectively known as microbiota, that reside in the human body are colonized in niches adjacent to epithelial surfaces of the gastrointestinal tract [16]. The diverse and abundant intestinal bacteria play a crucial role to the development and maturation of the immune system early in life, as well as in protection against pathogen colonization [17,18]. However, intestinal infection or colonization by pathogens or a pathobiont, and their released metabolites may alter the composition of gut microbiota [19,20].

Staphylococcus aureus persistently or intermittently colonizes the skin in the nasal area of ~50% of healthy adults, but it has emerged as a major early colonizer of the infantile gut [21,22]. Unlike *C. difficile*, there is limited data regarding the fecal carriage and intestinal colonization of methicillin resistant *S. aureus* (MRSA) among IBDs [23,24]. Thus, the main focus of this study was to estimate the prevalence of *C. difficile*, *S. aureus* and their coexistence in the gut of Iranian patients with IBD flare and remission.

2. Materials and methods

2.1. Patients and sample collection

Colonic biopsies and stool samples were collected from 70 IBD patients, who referred for the colonoscopy at Research Institute for Gastroenterology and Liver Diseases (RIGLD) in Tehran during September 2015 to June 2016. The definite diagnosis of IBD was made based on a combination of signs and symptoms, colonoscopic demonstrating pseudomembranous colitis and pathologic reports [25]. Clinical and demographic features were recorded for all patients through a questionnaire on the day of admission. The phase of disease including remission or flare was determined based on the diseases activity indices using Powell-Tuck index and Harvey-Bradshaw index for UC and CD patients, respectively [26,27]. The study protocol was approved by the Ethical Review Committee of RIGLD at Shahid Beheshti University of Medical Sciences (Project No. IR. SBMU.RIGLD.REC.1396.185). All experiments were performed in accordance with relevant guidelines and regulations recommended by the institution and informed consents were obtained from all subjects and/or their legal guardians prior to sample collection.

2.2. Bacterial culture

2.2.1. Stool samples

For isolation of *C. difficile*, stool samples were cultured using previously described methods [28]. Briefly, a spoonful of fecal sample (0.5–1 g) was mixed with 1 ml of 5% yeast extract broth and directly inoculated onto cycloserine-cefoxitin-fructose agar (CCFA) (Mast, UK), supplemented with 7% horse blood and *C. difficile* selective supplement (Mast, UK) composed of D-cycloserine (250 mg/L), cefoxitin (8 mg/L), and lysozyme (5 mg/L). The cultured plates were incubated at 37 °C for at least 48–72 h under anaerobic atmosphere (85% N_2; 10% CO_2 and 5% H_2) generated by Anoxomat® Gas Exchange System (Mart Microbiology BV, Holland). Additionally, the fecal samples were treated with 1 ml of methanol for 1–2 min before inoculation on the CCFA plates. The suspected organisms with characteristic colony morphology, Gram-positive staining, and occasionally sporulated bacilli were considered as *C. difficile*, and confirmed by PCR amplification of *cdd3* gene [28].

For the detection of *S. aureus*, a portion of homogenized stool samples were inoculated on mannitol salt agar (Merck, Darmstadt, Germany). Cultured plates were incubated at 37 °C for 24–48 h and colonies consistent with *S. aureus* were subjected to identification by colony morphology, Gram staining and standard biochemical tests [29].

Additionally, in order to rule out the presence of other enteric pathogens all samples were examined for *Campylobacter* spp., *Shigella* spp., *Salmonella* spp. *Yersinia enterocolitica*, and pathogenic *Escherichia coli* by culture on selective and specific media as previously described [30,31].

2.2.2. Colonic biopsies

The colon biopsies were transported to the laboratory in thioglycolate broth (Merck, Darmstadt, Germany) and homogenized with a suitable tissue grinder. Immediately, a hundred microliter of each homogenized biopsy was directly smeared on the surface of CCFA and MSA media. The culture plates were incubated at 37 °C under over-mentioned anaerobic and aerobic conditions for the culture of *C. difficile* and *S. aureus*, respectively.

2.3. DNA extraction and characterization of C. difficile and S. aureus isolates

Genomic DNA was extracted from the identified colonies of *C. difficile* by using InstaGene matrix extraction kit (Bio-Rad, USA) [32]. The purified DNAs were utilized to confirm the identification of *C. difficile* by amplification of *cdd3* gene, and to detect toxin A (*tcdA*), toxin B (*tcdB*) and binary toxin (*cdtA*, and *cdtB*) genes through PCR, as described previously [33,34]. A capillary gel-based electrophoresis PCR-ribotyping was performed for molecular typing of *C. difficile* isolates according to the consensus ribotyping protocol in the Department of Medical Microbiology, Motol University Hospital, Prague, Czech Republic [35]. The obtained CE-ribotyping profiles were compared with the patterns used in a CE-ribotyping validation study [35].

The *S. aureus* DNA was purified from overnight colonies using a modified boiling method, as previously described [36,37]. Briefly, colonies were suspended in 500 μl of Tris-EDTA buffer (10 mM Tris-HCL, 1 mM EDTA, pH = 8) followed by centrifugation for 2 min at 8000 g. The pellet was dissolved in 500 μl lysis buffer containing lysostaphin (100 mg/ml in sterile deionized water; Sigma), lysozyme (20 mg/ml) and proteinase k (10 mg/ml) and incubated for 10 min at 37 °C. Then, tubes were transferred to a boiling water bath for 20 min. After centrifugation (8000×*g*, 5 min), the supernatant containing the genomic DNA was kept at −20 °C until used for PCR. For molecular confirmation of *S. aureus* and detection of its enterotoxins, PCR amplifications were carried out for *femA*, *nucA* and *sea-see* genes, respectively [38–40]. The oligonucleotide primers and the expected sizes of their PCR products are shown in Table 1.

Table 1
The oligonucleotide sequences of the primers used in this study.

Agents	Target gene	Oligonucleotide sequence (5′–3′)	Product (bp)	References
***S. aureus* and enterotoxins**	*fem*	CTTACTTACTGCTGTACCTG ATCTCGCTTGTTGTGTGC	648	[38]
	nuc	GCGATTGATGGTGATACGGTT AGCCAAGCCTTGACGAACTAAAGC	270	
	sea	ATGGTTATCAATGTGCGGGTGIIIIICCAAACAAAAC TGAATACTGTCCTTGAGCACCAIIIIIATCGTAATTAAC	344	
	seb	TGGTATGACATGATGCCTGCACIIIIIGATAAATTTGAC AGGTACTCTATAAGTGCCTGCCTIIIIIACTAACTCTT	196	
	sec	GATGAAGTAGTTGATGTGTATGGATCIIIIIACTATGTAAAC AGATTGGTCAAACTTATCGCCTGGIIIIIGCATCATATC	399	
	sed	CTGAATTAAGTAGTACCGCGCTIIIIIATATGAAAC TCCTTTTGCAAATAGCGCCTTGIIIIIGCATCTAATTC	451	
	see	CGGGGGTGTAACATTACATGATIIIIICCGATTGACC CCCTTGAGCATCAAACAAATCATAAIIIIICGTGGACCCTTC	286	
***C. difficile* and toxins**	PS13 PS14	GGAGGCAGCAGTGGGGAATA TGACGGGCGGTGTGTACAAG	1062	[34]
	tcdA-F3345 tcdA-R3969	GCATGATAAGGCAACTTCAGTGGTA AGTTCCTCCTGCTCCATCAAATG	629	
	tcdB-F5670 tcdB-R6079A tcdB-R6079B	CCAAARTGGAGTGTTACAAACAGGTG GCATTTCTCCATTCTCAGCAAAGTA GCATTTCTCCGTTTTCAGCAAAGTA	410	
	cdtA-F739A cdtA-F739B cdtA-R958	GGGAAGCACTATATTAAAGCAGAAGC GGGAAACATTATATTAAAGCAGAAGC CTGGGTTAGGATTATTTACTGGACCA	221	
	ctdB-F617 cdtB-R878	TTGACCCAAAGTTGATGTCTGATTG CGGATCTCTTGCTTCAGTCTTTATAG	262	
	tcdA-F3345 tcdA-R3969	GCATGATAAGGCAACTTCAGTGGTA AGTTCCTCCTGCTCCATCAAATG	629	

2.4. Determination of antibiotic resistance

2.4.1. C. difficile

The antimicrobial susceptibility testing of *C. difficile* isolates was evaluated for 14 antimicrobials using agar dilution method. The following antibiotics were tested: metronidazole (MTZ), vancomycin (VAN), fidaxomicin (FDX), clindamycin (CLI), erythromycin (ERY), moxifloxacin (MXF), levofloxacin (LVX), ciprofloxacin (CIP), tetracycline (TET), rifampicin (RIF), chloramphenicol (CHL), ampicillin (AMP), meropenem (MEM) and imipenem (IPM) (Sigma-Aldrich, Germany). The breakpoints for most antimicrobial agents tested were according to CLSI criteria (document M11-A8) [41]. EUCAST version 8.0 (http://www.eucast.org) clinical breakpoints for *C. difficile* were applied to vancomycin. For fidaxomicin, erythromycin, ciprofloxacin, levofloxacin, and rifampicin, the previously published breakpoints were considered to interpret the results [42,43]. A series of two-fold serial dilutions of each antibiotic was made with the range of concentrations from 0.5 to 256 μg/ml. *C. difficile* ATCC 700057 was exploited as the control strain.

2.4.2. S. aureus

For the *S. aureus* isolates, resistance patterns to 11 antimicrobials including gentamicin (10 μg), amikacin (30 μg), ciprofloxacin (5 μg), vancomycin (30 μg), clindamycin (2 μg), trimethoprim/sulphamethoxazole (30 μg), penicillin (1 μg), amoxicillin-clavulanate (30 μg), chloramphenicol (30 μg), erythromycin (15 μg), and tetracycline (30 μg) (Padtanteb, Iran) were determined for by Kirby-Bauer disk diffusion method on Muller Hinton agar in accordance with CLSI recommendations [44]. MRSA was identified by growth on Mueller Hinton agar with 4% NaCl and oxacillin (6 μg/ml) [44,45]. *S. aureus* ATCC 25923 was used as a standard reference strain.

2.5. Statistical analysis

Data analysis was performed using SPSS software version 21 (SPSS Inc., USA). Statistical differences between the groups were analyzed by Fisher's exact test and the results were considered to be significant at a P value of <0.05.

3. Results

3.1. Characteristics of the patients

Totally, 70 stool samples and 70 colon biopsies were collected from IBD patients, consisting of 65 (92.9%) and 5 (7.1%) UC and CD patients, respectively. IBD remission was observed in 33.84% of UC (22/65) and 40% of CD (2/5) patients, and flares were seen in 66.16% of UC (43/65) and 60% of CD (3/5) patients. There were 34 (48.6%) male and 36 (51.4%) female patients, with the mean age of 33.6 ± 12.23 years (range 17–65 years). Clinical manifestations included watery diarrhea (25/70, 35.70%), loose stool (12/70, 17.1%), bloody stool (35/70, 50%), anorexia (13/70, 18.6%) and abdominal tenderness (24/70, 34.3%). The most commonly used drugs at the time of enrolment were ciprofloxacin (17/70, 24.3%), metronidazole (24/70, 34.3%), and ciprofloxacin + metronidazole (10/70, 14.3%). The extent and regions of disease involvement included 34.3% in the rectum, 31.4% in total colon, 17.1% in rectosigmoid, 14.3% in left-sided colon and 2.9% in transverse colon. Nine (12.9%) patients had severe disease, 3 (4.3%) had moderately severe disease, and 12 (17.1%) had mildly active disease. Demographic and clinical characteristics of the patients are shown in Table 2.

3.2. Prevalence of C. difficile and S. aureus in IBD flares

Overall, only four (5.7%) *C. difficile* isolates were cultured simultaneously from the stool and biopsy samples of patients with IBD flare. In addition, 13 (18.6%) *S. aureus* isolates were also detected in both stool and biopsy samples of IBD patients with flare. No enteric bacterial pathogens were detected in the remission phase. All *C. difficile*-infected patients had simultaneous colonization with *S. aureus* (100% concordance). Two different *C. difficile* ribotypes (RT

Table 2
Demographic data and clinical characteristics of the IBD patients.

Characteristics	UC (n = 65)		CD (n = 5)	
	Remission N (%)	Flare N (%)	Remission N (%)	Flare N (%)
Gender				
Female	13 (59)	19 (44.1)	1 (50)	1 (33.3)
Male	9 (41)	24 (55.9)	1 (50)	2 (66.7)
Age				
1–20	2 (9.1)	3 (7)	1 (50)	0
21–30	13 (59.1)	20 (46.5)	1 (50)	1 (33.3)
31–40	3 (13.7)	4 (9.3)	0	0
41–50	4 (18.1)	9 (21)	0	1 (33.3)
>50	0	7 (16.2)	0	1 (33.3)
Extent of disease				
Rectum	9 (41)	12 (27.9)	2 (100)	1 (33.3)
Rectosigmoid	4 (18.1)	8 (18.6)	0	0
Transverse colon	1 (4.5)	1 (2.3)	0	0
Total colon	6 (22.2)	14 (32.5)	0	2 (66.7)
Left-sided colon	2 (4.5)	8 (18.6)	0	0
Pathological findings				
Distortion of crypt architecture	10 (45.4)	20 (46.5)	1 (50)	2 (66.7)
Lymphoid aggregation	20 (90.9)	41 (95.3)	2 (100)	3 (100)
Lymphoplasmacytic infiltration	11 (50)	22 (51.1)	2 (100)	2 (66.7)
Tumor malignancy	0	1 (2.32)	0	0
Goblet cell depletion	0	5 (1.6)	0	19 (33.3)
Edema	1 (4.5)	4 (11.6)	0	0
Ulcer	0	2 (4.6)	0	0

UC, ulcerative colitis; CD, Crohn's disease.

126 n = 3 and RT 017 n = 1) were identified. The toxin profiles of RT 126 and RT 017 isolates were detected as $tcdA^+B^+/cdtA^+B^+$ and $tcdA^+B^+$, respectively. In *S. aureus* isolates, only positivity for the presence of *sea* enterotoxin was detected.

3.3. Antimicrobial susceptibility of the C. difficile and S. aureus isolates

The antibiotic susceptibility patterns of the *C. difficile* isolates were summarized in Table 3. As shown in the table, all isolates were susceptible to metronidazole, fidaxomicin and rifampicin. One isolate was found to be resistant to vancomycin with an MIC equal to 8 μg/ml. Moreover, all isolates were also found to be resistant to erythromycin (MIC ≥32 μg/ml), tetracycline (MIC ≥32 μg/ml), and ampicillin (MIC = 8 μg/ml).

The antibiotic resistance patterns of *S. aureus* isolates were shown in Fig. 1. The highest resistance was observed against penicillin (12/13, 92.3%), following amoxicillin-clavulanate (5/13, 38.5) and amikacin (4/13, 30.8). All isolates were susceptible to chloramphenicol and vancomycin. MRSA and multi-drug-resistant *S. aureus* (MDRSA) were detected in 2/13 (15.4%) and 3/13 (23.1%) of the isolates investigated.

4. Discussion

In recent years, the incidence of IBD was extraordinary on rise and doubled every decade. This disabling disease can impose a substantial burden on health-care systems in many countries worldwide [46]. The interplay between gut microbiota and host immune cells plays a key role in instruction and regulation of the mucosal immunity. Thus, abnormal and abrogated microbial communities, called intestinal dysbiosis, may dysregulate the mucosal immune responses in the gut of IBD patients. Furthermore, patients with IBD are frequently hospitalized and are at a higher risk developing opportunistic and antibiotic-resistant infections [24]. The presence of hospital-acquired infections exacerbate the

Table 3
Interpretive criteria and MIC values of 14 antibiotics tested for *C. difficile* isolates.

Antibiotics	Breakpoints (μg/ml)			No. of isolates for each MIC value 0.5–256 (μg/ml)										Resistance interpretation		
	S	I	R	0.5	1	2	4	8	16	32	64	128	256	S	I	R
Metronidazole	≤8	16	≥32	4										4	0	0
Vancomycin	≤2	NA	>2	2	1			1						3	0	1
Fidaxomicin	<1	>1	NA	4										4	0	0
Imipenem	≤4	8	≥16			1		3						1	3	0
Meropenem	≤4	8	≥16	1						1	2			1	0	3
Erythromycin	NA	NA	≥8							1	1	1	1	0	0	4
Moxifloxacin	≤2	4	≥8	1			1			2				1	1	2
Ciprofloxacin	NA	NA	≥8				1	1		1			1	1	0	3
Levofloxacin	NA	NA	≥8	1					1		1	1		1	0	3
Tetracycline	≤4	8	≥16							2	2			0	0	4
Rifampicin	NA	NA	≥32					4						4	0	0
Chloramphenicol	≤8	16	≥32					2		2				2	0	2
Ampicillin	≤0.5	1	≥2					4						0	0	4
Clindamycin	≤2	4	≥8	2									2	2	0	2

Breakpoints were defined as susceptible (S), intermediately resistant (I), or resistant (R) with reference to CLSI, EUCAST or published data. NA, not available.

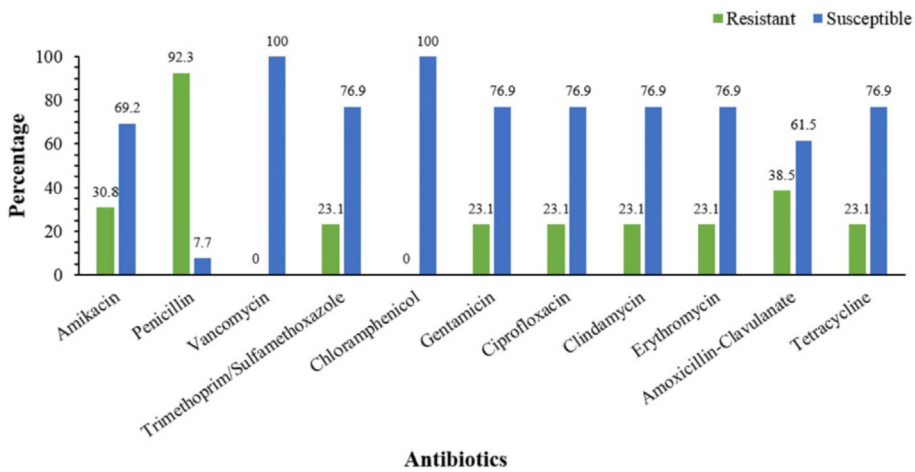

Fig. 1. Antimicrobial susceptibility patterns of *S. aureus* isolates from IBD patients.

intestinal barrier damage and intensify the acute phase of disease. This disruption in the function of intestinal epithelial barrier alongside with inadequate antibiotic prescription, and poor adherence to infection control guidelines may increase the risk of antibiotic-associated infections among IBD patients, specifically CDI [15]. Also, it has been reported that hospitalized IBD patients are at increased risk of MRSA compared with non-IBD gastrointestinal and general medical inpatients [24]. In the context of IBD, the presence of these infections was found to trigger an acute disease flare and are associated with increased rates of hospitalization and mortality [8].

In the present study, 70 IBD patients were involved for investigation of the prevalence of *C. difficile* and *S. aureus* and their coexistence among patients with IBD flare and remission. Our results showed that *C. difficile* was detected in 5.7% of the patients with IBD flare. Accordingly, our data demonstrated a lower rate of CDI in IBD patients than previously published studies [47–49]. A potential explanation for the lower CDI rate compared to others is that the majority of enrolled IBD patients identified as outpatients in our study. However, our results were consistent with a previous report from a retrospective and prospective cohort study performed in the Netherlands, where toxigenic *C. difficile* was detected in 3.6% of consecutive IBD outpatients [50].

In recent years, multiple observational studies have revealed a relationship between enteric infections, altered microbiota, and the subsequent development of IBD [48,51,52]. A number of different enteric infections caused by bacterial, viral, fungal, protozoal, and helminthic pathogens have been reported to cause similar symptoms as seen in exacerbation of IBD [53]. Of these infectious agents, *C. difficile* has been considered to be the most common (12.9%) enteric infection with increasing CDI rates in patients with IBD [14,48,49]. In addition, IBD patients not only have a higher prevalence of CDI, but also significantly show worse clinical outcomes with increased morbidity and mortality [47,54]. Given the critical importance of CDI in patients with IBD, early detection of this infection is crucial to minimize the adverse outcomes.

As over-mentioned, several non-*C. difficile* related enteric infections including *Campylobacter*, *Escherichia coli* species, *Plesiomonas*, and Norovirus have been reported to mimic the IBD outcomes, and some of them may play an important role in flare of IBD [48,52]. Furthermore, Nguyen et al. reported that increased prevalence of MRSA in the IBD patients to be 1.4-fold higher than that of general patients, and that this was associated with a greater than 7-fold increase in hospital mortality [23]. However, the impact of CDI co-infections with aforementioned enteric infections on clinical outcomes of IBD patients is not well studied. *S. aureus* was detected with a higher rate (18.6%) than *C. difficile* in our cohort of patients. Our results also showed that 5.7% of IBD patients with CDI were co-infected with the enterotoxigenic *S. aureus* isolates (sea^{+}). Interestingly, all *C. difficile*-infected patients were simultaneously colonized with *S. aureus* (100% concordance). To best of our knowledge, this is the first report on coexistence of *C. difficile* and *S. aureus* among patients with IBD. These findings also suggests that the presence of *C. difficile* may increase the risk of accruing community-acquired MRSA (CA-MRSA) in IBD outpatients. Moreover, it has been proposed that gastric acid suppression with PPIs or H_2-receptor blockers was associated with increased risk of MRSA colonization in the ambulatory IBD population [23]. The use of acid-suppressive therapy, particularly PPIs has been also associated with other infections such as community-acquired *C. difficile* [55,56]. Therefore, suppression of acid secretion likely increases co-colonization and overgrowth of opportunistic bacterial species from the oral and nasal cavity in the gastrointestinal tract, which may include MRSA [23,57].

5. Conclusions

In conclusion, our findings demonstrated that patients with IBD flare are more vulnerable to acquire coinfection of *C. difficile* and *S. aureus* than remission not only in the hospital setting, but also in the community. Therefore, we recommend clinicians to be vigilant about considering the coinfection of CDI and *S. aureus* in symptomatic IBD patients, particularly in the community care setting. However, more robust data is required to investigate the interaction between these enteric infections, their clinical relevance and outcomes in patients with IBD flare. Taken together, this will help address the appropriate treatment strategies of IBD patients in whom relapse is complicated by enteric coinfection.

Declaration of competing interest

The authors declare that they have no conflicts of interest.

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