In vitro susceptibility of clinical Clostridioides difficile isolates to ridinilazole and ibezapolstat in Israel, 2020-2022

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Abstract

Background

Even though they constitute a risk factor, antibiotics are still the current primary treatment of *C. difficile* infection. Due to *C. difficile*'s rapid development of resistance and high recurrences rates, there is an unmet need for new antimicrobials. In the current study, we assessed the in vitro susceptibility of clinical isolates from Israel to two recently developed antibiotics, ridinilazole and ibezpolstat, and to currently used antibiotics.

Methods

We collected 313 *C. difficile* isolates from several medical centers across Israel, that were recovered from patients of both community and hospital facilities. Isolates were typed to different strains (strain type-ST) by multi-locus sequencing typing (MLST). Susceptibility to metronidazole and vancomycin was determined by Etest; susceptibility to fidaxomicin, ridinilazole and ibezpolstat was determined by agar dilution.

Results

The most prevalent STs were ST42, with 39 (12.5%) isolates and ST2, with 36 isolates (11.5%). Resistance rate to metronidazole and vancomycin was low (2.2%, 1.6%, respectively). Ridinilazole (RDZ) MIC ranged between 0.06 to 0.5 mg/L, and the $MIC_{50/90}$ were 0.25/0.5 mg/L. Ibezpolstat (IBZ) had an $MIC_{50/90}$ of 4 mg/L. No significant differences were noted in MIC of different strains.

Conclusions

We demonstrated a potent *in vitro* activity of RDZ and IBZ against 313 *C. difficile* isolates, belonging to different STs. These two antimicrobials may serve as a treatment for *C. difficile* infection, as they have an excellent activity against *C. difficile* on one hand, and minimal effect on gut microbiome, on the other hand.

Background

Clostridioides difficile (*C. difficile*) is a Gram-positive, anaerobic bacterium which causes significant diarrheal illness, both in healthcare and community facilities (1).

Since 2011, Emerging Infections Program (EIP) of the Centers for Disease Control and Prevention (CDC) has been monitoring *C. difficile* infection (CDI) in 10 US sites. A recent study which used the EIP data, reported on a 24% decrease in the total burden of CDI in the US between the years 2011 and 2017.

Nevertheless, there was no changes in the high burden of first recurrences and of CDI-associated in-hospital mortality (2).

CDI onset begins with ingestion of spores, followed by their germination in the gut, which results in bacterial colonization and proliferation. Then, toxins produced by the bacteria disrupt the gut epithelial integrity, induce cytotoxic effects on intestinal cells and stimulate an inflammatory response (1). The main risk factor for developing CDI is antibiotic administration as it induces dysbiosis, i.e., alteration of the gut microbiome composition, which results in germination of *C. difficile* spores (3). Another potential outcome of dysbiosis is an increased ratio of primary-to-secondary bile acids. As primary bile acids promote spore germination while secondary bile acids inhibit *C. difficile* growth, this shift also contributes to CDI development (3).

Even though they constitute a risk factor, antibiotics are currently the primary treatment for CDI. The main options in use are fidaxomicin (FDX) and vancomycin (VAN); metronidazole may be given when the previous two antibiotics are not available (4). Although MTZ and VAN are effective against vegetative *C. difficile* cells, their use still induces gut microbiome disruption, which may lead to further *C. difficile* spore germination and disease recurrence (5). FDX advantages over VAN and MTZ, are the lower concentration needed for treatment, longer duration of effect, and reduced disease recurrence rate (6). Additionally, FDX use has been associated with less microbiome dysbiosis (6).

In recent years, several new *C. difficile* strains have emerged, with some of them being resistant to antibiotics in clinical use. Additionally, up to 30% of treated patients may experience recurrent CDI due to persistence of antibiotic-resistant spores (7). Thus, new alternatives are required. One of the newest developed antibiotics for CDI treatment is ridinilazole (RDZ), a narrow-spectrum antibiotic. RDZ is a a bis-benzimidazole antibiotic with a bacterricidal activity that is mediated by its interaction with AATTT-rich sequences in *C. difficile* DNA minor groove, leading to interference with cell division and apparently with ATP production (8).

In addition to its high inhibitory activity against several *C. difficile* strains, both *in vitro* and *in vivo* (9, 10), RDZ reduced CDI recurrence rate from 17.3–8.1%, compared to VAN ($p = 0.0002$) (8). In contrast to VAN, RDZ did not impact the gut microbiome(11, 12) and had no effect on secondary bile acids (12). In line with these reports, two clinical trials (NCT03595553 and CT03595566) comparing the efficacy of RDZ vs. VAN, found better conservation of gut microbiome with RDZ (8). RDZ treatment in a phase 3 superiority trial was associated with less recurrence cases and increased secondary bile acids levels (8). Recently, a phase 3 study of RDZ has been terminated; due to the insuperiority of RDZ on VAN in sustained clinical response (73%, and 70.7%, respectively), the drug company which developed RDZ stated on rethinking(8)

Ibezapolstat (IBZ) is another treatment recently developed for CDI. This narrow-spectrum antibiotic binds and inhibits DNA polymerase IIIC (DNA pol IIIC), which is unique to Gram-positive bacteria with a low G + C content. IBZ has been tested in clinical trials and has exhibited several advantages, including minimal adverse effects, good pharmacokinetics, a favourable secondary-to-primary bile acid ratio and

limited damage to the gut microbiome (13). Murray et al. reported on the potent activity of IBZ against 104 *C. difficile* isolates (14). Recently, phase 2b clinical trial of ibezapolstat has been completed.

To the best of our knowledge, there are no data regarding susceptibility of clinical *C. difficile* strains to RDZ and IBZ in Israel. Additionally, since antibiotic susceptibility testing for *C. difficile* is not routinely evaluated by clinical laboratories, there are limited data on susceptibility rates to the currently used treatments. Therefore, this study aims to assess the susceptibility of different clinical strains, collected from several areas in Israel between 2020 and 2022, to RDZ, IBZ, FDX, MTZ and VAN.

Methods

Study isolates

The study included 313 *C. difficile* isolates that were recovered from stool samples of patients diagnosed with CDI and hospitalized in one of four medical centres in Israel between 2020 and 2022. CDI was confirmed with the GeneXpert *C. difficile* BT PCR assay (Cepheid, Sunnyvale, CA, USA) which identifies toxin B and binary toxin genes, and *tcd*C deletion (independent identification of the epidemic Nap1/027 strain). Community-acquired CDI (CA-CDI) was defined as CDI that developed within 48 hours of admission, while hospital-acquired CDI (HA-CDI) was defined as CDI that developed > 48 h after admission.

The four participating medical centres are located in different geographic areas of Israel: North - Tzafon Medical Center, Poriya and W. Hirsch Regional Microbiology Laboratory Clalit Health Services, Haifa, Center - Edith Wolfson Medical Center, Holon, and South - Soroka University Medical Center, Be'er Sheva. The local Ethics (Helsinki) Committee of each medical centre approved the study (POR-0085-15, WOMC-0115-20, SOR-0307-20). The need for informed consent was waived.

Multi-locus sequence typing (MLST)

DNA was extracted from study isolates using the MagCore® Genomic DNA Bacterial Kit (ATRIDAB.V, Amersfoort, Netherlands) with the MagCore® automated extraction instrument (RBCBioscience, New Taipei, Taiwan), according to the manufacturer's instructions. Following whole-genome sequencing of DNA samples, the sequences of seven housekeeping genes (*adk*, *atp*A, *dxr*, *gly*A, *rec*A, *sod*A, and *tpi*) of each isolate were uploaded to the *C. difficile* MLST database (https://pubmlst.org/organisms/clostridioides-difficile), in order to determine the sequence type (ST), as previously described (15).

Bacterial isolation and identification

Stool samples were inoculated on a selective growth agar medium, chromID™ *C. difficile* (CDIF) (BioMérieux, Durham, NC), and incubated for 48 h, at 37°C, in a Bactron EZ 300 anaerobic chamber

(Sheldon manufacturing, Cornelius, USA). Identification of *C. difficile* colonies was based on colonies' asymmetrical shape and black color. The Bruker Biotyper system (Bruker Daltonics, Bremen, Germany), that is based on matrix-assisted laser desorption ionization-time of flight (MALDI-TOF) mass spectrometry, was used for definitive. All isolates were stored at -80°C until further analysis.

Antimicrobial susceptibility testing

Susceptibility testing for MTZ and VAN

Susceptibility to MTZ and VAN was assessed using the Etest technique, which determines the minimum inhibitory concentration (MIC). Several *C. difficile* colonies were suspended in thioglycollate broth medium (Becton Dickinson, Heidelberg, Germany) to achieve 1.0 McFarland standards. Then, bacterial suspensions were inoculated on Brucella blood agar (Hy Laboratories, Rehovot, Israel) and a gradient Etest strip (bioMérieux, Durham, NC) of VAN or MTZ was added. The agar plates were incubated at 37°C under anaerobic conditions for 48 h. Following incubation, the MIC was visually determined and isolates were classified as susceptible or resistant according to breakpoints of the European Committee on Antimicrobial Susceptibility Testing (EUCAST) guidelines (16).

Susceptibility testing for FDX, RDZ and IBZ

Susceptibility to FDX, RDZ and IBZ in accordance with the procedures of the Clinical and Laboratory Standards Institute (CLSI-M11-9th) (17). Brucella agar supplemented with 5% defibrinated sheep blood (Hy Laboratories) was mixed with FDX (Sigma-Aldrich, Missouri, US), RDZ or IBZ (MedChemExpress LLC, NJ, USA) by first dissolving the antibiotics in dimethyl sulfoxide (DMSO) and then further diluting it with distilled water to various concentrations. The agar plates were mixed with the different dilutions of each antibiotic, yielding the following ranges of final concentrations: FDX 0.03-32 mg/L, RDZ-0.03-0.5 mg/L, and IBZ- 0.5-8 mg/L.

Several *C. difficile* colonies were inoculated in thioglycollate broth medium (Becton Dickinson) to 0.5 McFarland turbidity, and then placed as spots on the antibiotics-supplemented agar plates. Plates were incubated at 35°C, under anaerobic conditions, for 48 h. After incubation, plates were visually screened for bacterial growth, and MIC was determined as the lowest antibiotic concentration that inhibited bacterial growth.

Results

The study included 313 isolates, recovered from 187 patients with HA-CDI and 126 patients with CA-CDI.

ST distribution

ST was determined for 90.1% (282/313) of the isolates, while the rest of isolates remained unclassified. Isolates were categorized into ten major groups, with each group containing at least seven isolates (Table 1). An additional group, called "others", included 108 (34.5%) isolates had STs shared by fewer than seven isolates (Supplemental Table 1).

Table 1
Distribution of ST among study isolates

ST	Clade	n (%)
ST42	1	39 (12.5)
ST2	1	36 (11.5)
ST104	1	23 (7.3)
ST11	5	21 (6.7)
ST3	1	13 (4.2)
ST34	1	9 (2.9)
ST54	1	9 (2.9)
ST55	1	9 (2.9)
ST37	4	8 (2.6)
ST13	1	7 (2.2)
Others	1, 2, 3, 4	108 (34.5)
Unclassified	N.A.	31 (9.9)

The most prevalent STs were ST42 (n = 39; 12.5%) and ST2 (n = 36; 11.5%). Most isolates (274/282; 97.2%) belonged to Clade 1, one isolate with ST1 belonged to Clade 2, 2 isolates with ST5 belonged to Clade 3, 9 isolates (8 ST37 and 1 ST39), belonged to Clade 4 and 21 isolates of ST11, belonged to Clade 5. The "Others" group included isolates from Clades 1–4 (Table 1). One hypervirulent strain (0.3%), belonged to ST1, was found.

Overall, the same STs were found among both CA and HA isolates, however, their distributions differed (Fig. 1). For example, among HA isolates, ST42 (13.9%) was the most common strain, while ST2 was the most (13.5%) frequent ST among CA isolates. ST37 was more frequent detected among HA isolates compared to CA isolates (3.7%, 0.8%, respectively).

Susceptibility of study isolates

Bacterial susceptibility to MTZ, VAN, FDX and to the new antimicrobials RDX and IBZ was assessed (Table 2, Fig. 2). The MIC of MTZ were in the range of 0.016-256 mg/L and $MIC_{50/90}$ were 0.19/0.38 mg/L. Resistance rate to MTZ was low (2.2%). The MIC_{50} of VAN was 0.5 mg/L, the MIC_{90} was 0.75

mg/L, and the resistance rate was low (1.6%). The geometric MIC means of both MTZ and VAN were quite high (6 mg/L and 2.5 mg/L, respectively). FDX MIC was in the range of 0.03-16 mg/L and $MIC_{50/90}$ was 0.25/0.5 mg/L. Twenty three (7.35%) isolates were resistant to FDX. RDX MIC ranged between 0.06 mg/L and 0.5 mg/L, and the $MIC_{50/90}$ was 0.25/0.5 mg/L. IBZ had an $MIC_{50/90}$ of 4 mg/L.

Table 2
Antimicrobial susceptibility of study isolates

Antimicrobial agent	MIC Range (mg/L)	MIC_{50} (mg/L)	MIC_{90} (mg/L)	Geometric MIC mean (mg/L)	% Resistance
Metronidazole	0.016-256	0.19	0.38	6	2.2
Vancomycin	0.064-256	0.5	0.75	2.5	1.9
Fidaxomicin	0.03-16	0.25	0.5	0.81	7.35
Ridinilazole	0.06–0.5	0.25	0.5	0.32	N.A.
Ibezapolstat	0.5-8	4	4	3.1	N.A.

Different STs had no major differences in MIC to the different antibiotics, including RDX and IBZ (Table 3). Furthermore, when we compared the MIC_{50}/90 and geometric mean MIC values for RDZ and IBZ between MTZ-susceptible and MTZ-resistant strains, as well as between VAN-susceptible and VAN-resistant strains, no differences were observed; similar result was seen for FDX- susceptible and FDX-resistant strains (Table 4). We also compared our results with data from all studies that tested the *in vitro* activity of RDZ and/or IBZ (Table 5). RDZ MIC in the current analysis were among the highest reported values. In most studies, RDZ had a lower $MIC_{90,}$ compared to MTZ and VAN. When compared to the newest antibiotic in use, RDZ had either lower or equal MIC_{90}.

Table 3
Antibiotic susceptibility of study isolates, with relation to ST

Strains	MIC (µg/mL)	MTZ	VAN	FDX	RDZ	IBZ
ST2	Range	0.032-1	0.094-2	0.03-16	0.12–0.5	2–4
(n = 36)	MIC_{50}	0.125	0.5	0.25	0.25	4
	MIC_{90}	0.25	0.75	0.25	0.5	4
ST3	Range	0.023-0.2	0.25–0.75	0.03-16	0.12–0.25	1–4
(n = 13	MIC_{50}	0.094	0.75	0.12	0.25	4
	MIC_{90}	0.25	0.75	0.25	0.5	4
ST11	Range	0.016-256	0.064-256	0.03-16	0.12–0.5	2–4
(n = 21)	MIC_{50}	0.094	0.75	0.12	0.5	2
	MIC_{90}	0.25	1	0.25	0.5	4
ST13	Range	0.125–0.25	0.5–0.75	0.06-16	0.12–0.5	2–4
(n = 7)	MIC_{50}	0.19	0.5	0.25	0.5	4
	MIC_{90}	0.25	0.75	0.25	0.5	4
ST34	Range	0.032–0.38	0.5-3	0.06-8	0.06–0.5	2–4
(n = 9)	MIC_{50}	0.19	0.75	0.25	0.25	4
	MIC_{90}	0.38	0.75	0.25	0.5	4
ST37	Range	0.016-256	0.5–0.75	0.06–0.25	0.06–0.5	1–4
(n = 8)	MIC_{50}	0.19	0.75	0.25	0.25	4
	MIC_{90}	0.75	0.75	0.25	0.5	4
ST42	Range	0.016-256	0.25-56	0.03-4	0.06–0.5	0.5-4
(n = 39)	MIC_{50}	0.25	0.75	0.25	0.25	4
	MIC_{90}	0.75	0.75	0.25	0.5	4
ST54	Range	0.023–0.38	0.38-1	0.06–0.25	0.12–0.5	1–4
(n = 9)	MIC_{50}	0.19	0.75	0.25	0.25	2
	MIC_{90}	0.25	1	0.25	0.5	4

Strains	MIC (µg/mL)	MTZ	VAN	FDX	RDZ	IBZ
ST55	Range	0.064–0.38	0.5–0.75	0.12–0.25	0.25–0.5	4–8
(n = 9)	MIC_{50}	0.125	0.75	0.25	0.25	4
	MIC_{90}	0.25	0.75	0.25	0.5	4
ST104	Range	0.023-1	0.38-1	0.03-16	0.06–0.5	1–4
(n = 23)	MIC_{50}	0.19	0.5	0.25	0.25	2
	MIC_{90}	0.25	1	0.5	0.5	4
Unclassified	Range	0.047-256	0.125-2	0.06-16	0.06–0.5	1–4
(n = 31)	MIC_{50}	0.19	0.5	0.25	0.5	2
	MIC_{90}	0.25	0.75	8	0.5	4
Others	Range	0.016-256	0.125-256	0.03-16	0.06–0.5	0.5-8
(n = 108)	MIC_{50}	0.19	0.75	0.25	0.25	2
	MIC_{90}	0.38	0.75	0.25	0.5	4

Table 4
Susceptibility of study isolates to RDZ and IBZ, with relation to their susceptibility to MTZ, VAN and FDX

Antimicrobial agent	RDZ			IBZ		
	MIC_{50}	MIC_{90}	Geometric MIC mean	MIC_{50}	MIC_{90}	Geometric MIC mean
(mg/L)						
Metronidazole-S	0.5	0.5	0.313	4	4	3.07
Metronidazole-R	0.5	0.5	0.41	4	4	3
Vancomycin-S	0.25	0.5	0.314	4	4	3.07
Vancomycin-R	0.5	0.5	0.374	4	4	3.2
Fidaxomicin-S	0.25	0.5	0.31	4	4	3.06
Fidaxomicin-R	0.5	0.5	0.37	4	4	3.21

Table 5
Summary of reported data regarding *C. difficile* susceptibility to MTZ, VAN, FDX, RDZ and IBZ

Reference (Geographic area)	No. of isolates	MIC (μg/mL)	IBZ	RDZ	FDX	VAN	MTZ
Dvoskin et al., 2012 (USA) [36]	23	Range	Not shown	N.D.	N.D.	N.D.	N.D.
		MIC_{50}	2				
		MIC_{90}	4				
Goldstein et al., 2013 (USA) [34]	50	Range	N.D.	0.125-0.5	0.06-1	1–8	0.25-8
		MIC_{50}		0.25	0.25	1	0.5
		MIC_{90}		0.25	0.5	4	2
Corbett at al., 2015 (UK) [37]	82	Range	N.D.	0.06–0.125	0.008–0.125	0.5-4	0.125-8
		MIC_{50}		0.125	0.03	1	2
		MIC_{90}		0.125	0.06	2	8
Freeman et al., 2016 (Europe) [38]	107	Range	N.D.	0.015-0.5	0.004–0.125	0.5-8	< 0.125-2
		MIC_{50}		0.03	0.06	1	0.2
		MIC_{90}		0.125	0.125	2	2
Snydman et al., 2017 (US) [39]	200	Range	N.D.	0.12–0.5	0.015-1	0.25-4	0.12-2
		MIC_{50}		0.12	0.03	1	0.25
		MIC_{90}		0.25	0.125	2	1
Snydman et al., 2018 (US) [11]	44	*Range	N.D.	0.06–0.5	0.06-1	1–4	0.12-4
		MIC_{50}		0.12	0.12	1	0.5
		MIC_{90}		0.25	0.5	2	2
	45	#Range		0.06–0.5	0.06-1	0.5-2	0.12-2

N.D., not detected

Reference (Geographic area)	No. of isolates	MIC (µg/mL)	IBZ	RDZ	FDX	VAN	MTZ
		MIC_{50}		0.12	0.25	1	0.25
		MIC_{90}		0.5	0.5	2	1
van Eijk, et al., 2019 (the Netherlands) [33]	363	Range	0.5-4	N.D.	N.D.	N.D.	N.D.
		MIC_{50}	2				
		MIC_{90}	4				
Murray et al., 2020 (USA) [14]	104	Range	1–8	N.D.	0.015-1	0.5-4	0.25-16
		MIC_{50}	4		0.12	1	0.5
		MIC_{90}	4		0.25	2	1
Collins et al., 2021 (Japan, China, South Korea) [30]	140	Range	N.D.	0.03–0.25	0.015–0.25	0.06-4	0.06–0.5
		MIC_{50}		0.125	0.125	1	0.25
		MIC_{90}		0.25	0.25	2	0.25
Snydman et al., 2023 (US) [29]	300	Range	N.D.	0.3–0.5	0.03–0.5	0.25-4	0.12-4
		MIC_{50}		0.25	0.25	2	0.5
		MIC_{90}		0.25	0.5	2	1
Bassères et al., 2024 (US) [32]	100	Range	-	N.D.	-	-	-
		MIC_{50}	4		0.5	2	0.25
		MIC_{90}	8		1	4	4
The current study (Israel)	313	Range	0.5-8	0.06–0.5	0.03-16	0.064-256	0.016-256
		MIC_{50}	4	0.25	0.25	0.5	0.19
		MIC_{90}	4	0.5	0.5	0.75	0.38
N.D., not detected							

IBZ $MIC_{50}/_{90}$ in the current study were similar to those reported in a recent study from the USA (Table 5). Out of the four studies evaluating IBZ, only two, including the current study, tested other antibiotics as well. In these two studies, IBZ MIC_{90} was higher than the VAN, MTZ and FDX MIC_{90}.

Discussion

The current study investigated the susceptibility of 313 *C. difficile* isolates collected from patients across Israel, to the recently developed antimicrobials RDZ and IBZ, as well as to currently used antibiotics.

ST distribution

ST42 and ST2 were the predominant (12.5%, 11.5%, respectively) STs in the current study. ST42 which correlates to Ribotype106/RT106, has been reported worldwide. In the US, RT106 became the second most detected strain in 2012 and the most prevalent strain in 2016 (18, 19). This strain produces both toxins A and B, but not the binary toxin. Additionally, it showed high resistance to erythromycin, clindamycin, fluoroquinolones, and third-generation cephalosporins. Most RT106 isolates are susceptible to metronidazole and vancomycin (19).

Regarding ST2, a study performed in a Chinese hospital found ST2 to be the second-most-prevalent ST (10, 11.11%) among the 90 isolated strains (20). Similar to RT106/ST42, ST2 is toxins A and B producer (20). A recent study from Germany that characterized *C. difficile* isolates recovered from various environmental samples, also found ST2 to be the second-most-prevalent (14/166, 8%) ST (21). Although the contribution of environmental bacteria to CDI is not fully understood, the similar prevalence of ST2 in both clinical and environmental isolates suggests that the environmental strains constitute a source for both CA- and HA-CDI.

Interestingly, a previous study conducted by our group, which characterized 70 *C. difficile* isolates during the years 2016–2018, reported ST4 (22.5%) and ST37 (12.7%) were the most common STs (22). Although this study was performed in one geographic area in Israel, the different distribution of STs as compared to the current study suggest the epidemiology of CDI have been changing in Israel. Additionally, studies from other Middle East countries reported different strain distribution; for example, An Iranian study which investigated 65 isolates in 2020, found that RT001/ST3 (32.3%) and RT126/ST11 (9.2%) were the most prevalent strains (23). In Lebanon, two strains were the dominant in 2018- RT014/ST2 (16.8%), which is similar to our results, and RT002/ST8 (9.3%) (24). Thus, *C. difficile* strains' distribution differs in different geographic areas and different periods.

Among our HA isolates, ST42 was more common than ST2. In contrast, the opposite order was seen among CA-CDI isolates, where ST2 was the most detected strain, followed by ST42. Several studies suggested that RT014/RT020/ST2 has a community origin (25, 26), a hypothesis that was strengthened by the current results. Furthermore, RT014/ST2 isolates have been recovered from various environmental sources including wastewater (27), parks and homes (28), another strengthening evidence for a community origin for this strain.

Susceptibility of study isolates to antibiotics in clinical use

Resistance rates to MTZ and VAN were low (2.2% and 1.6%, respectively). A previous study by our group, which characterized 70 isolates from north Israel, reported on higher (17.1%) resistance rate to MTZ and a similar resistance rate (1.4%) to VAN (22). As mentioned earlier, these changes may indicate on the evolving epidemiology of *C. difficile* in Israel. Lower resistance rates to both MTZ (0.3%) and VAN (0.7%) were reported in a recent US study of 300 *C. difficile* isolates (29). Collins et al, who investigated the susceptibility of 140 *C. difficile* isolates from Japan, China and South Korea, to various antibiotics, did not find any isolates resistant to either MTZ or VAN (30). Our low resistance rates might result from decreased use of MTZ and VAN in recent years, due to the introduction of FDX into clinical use.

FDX MIC_{50} in the current study was low (0.25 µg/mL). However, high FDX MICs were detected (the highest MIC was 32 µg/mL), as compared to MICs reported in recent studies (see Table 5). Although there is no clinical breakpoint for FDX, using the 0.5 mg/L cut-off proposed by EUCAST suggests that there are already FDX-resistant strains in Israel. Yet, according to a recent systematic review and meta-analysis of 1184 isolates, the resistance rate to FDX based on a breakpoint of ≥ 8 mg/L, was 0.08% (31). Thus, FDX susceptibility should be further monitored for early recognition of resistant strains and treatment failure.

Susceptibility of study isolates to RDZ and IBZ

Overall, low MIC to RDZ and IBZ were observed. Additionally, their activities were efficient against all STs and against isolates with different susceptibilities to MTZ/VAN/FDX. Specifically for IBZ, our results strengthen previous reports according to which the activity of IBZ was not different against different ribotypes or strains with different MTZ/VAN/FDX susceptibility patterns (32, 33).

When comparing the present results with recent studies that investigated RDZ susceptibility, the MICs in the current study were among the highest reported values. For example, most studies reported on MIC_{90} of 0.25 mg/L and lower, while in the current study, the MIC_{90} was 0.5 mg/L. Currently, there are no breakpoints for this new antimicrobial and further studies should be performed to gain sufficient and a comprehensive data regarding *C. difficile* susceptibility to RDZ.

Comparison of RDZ activity to that of other antibiotics showed that the RDX MIC_{90} was generally lower or similar to the MIC_{90} of FDX and always lower than those of VAN and MTZ. Furthermore, RDZ was shown to have lesser activity against Gram-negative anaerobes and Gram-positive aerobes, as compared to FDX, VAN and MTZ (34). Thus, the superiority of RDZ over the currently used antibiotics is expressed not only by its increased potency against *C. difficile*, but also by its reduced effect on the gut microbiome. It should be noted that RDZ's manufacturer has stopped clinical trials due to the non-superiority of RDZ on VAN in sustained clinical response and consider changes in this molecule (35).

The IBZ MIC_{50} and MIC_{90} for isolates in the current study aligned with those previously reported by others. However, only few studies reported on *in vitro* susceptibility of *C. difficile* to this antimicrobial. Thus, additional studies are needed in order to have a good comparison of susceptibility of isolates from different geographic areas.

Of note, IBZ had a wider MIC range and higher values of $MIC_{50/90}$, as compared to RDZ, which may indicate increased potency of RDZ as compared to IBZ. However, our study is the only study that tested both RDZ and IBZ activity and thus we do not have previous data to compare with, and this issue should be further investigated.

IBZ MIC was also higher than those of FDX, MTZ and VAN. However, the main advantages of IBZ include minimal adverse effects and limited interruption to gut microbiome (13), which outweigh the high dose requirement.

Conclusions

This study demonstrated the potent *in vitro* activity of RDZ and IBZ against 313 *C. difficile* isolates belonging to different STs and clades. So far, the two antimicrobials has proved to be ideal for CDI treatment, with excellent activity against *C. difficile* and minimal impact on bacterial species that constitute the gut microbiome.

Abbreviations

CA: community-acquired; CDI: *C. difficile* infection; FDX: fidaxomicin; HA: hospital-acquired; IBZ: ibezpolstat; MALDI-TOF: matrix-assisted laser desorption ionization-time of flight; MIC: minimum inhibitory concentration; MLST: multi-locus sequencing typing; MTZ: metronidazole; RDZ: ridinilazole; ST: sequence type; VAN: vancomycin

Declarations

Ethics approval and consent to participate: The local Ethics (Helsinki) Committee of each medical centre approved the study (POR-0085-15, WOMC-0115-20, SOR-0307-20). The need for informed consent was waived.

Clinical trial number: not applicable

Consent for publication: Not applicable

Availability of data and materials: The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Competing interests: The authors declare that they have no competing interests.

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Authors' contributions: OS collected the data, performed the experiments, analysed results and wrote the manuscript. MA and AP were major contributors in result analysis and the manuscript writing. All

authors read and approved the final manuscript.

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Figures

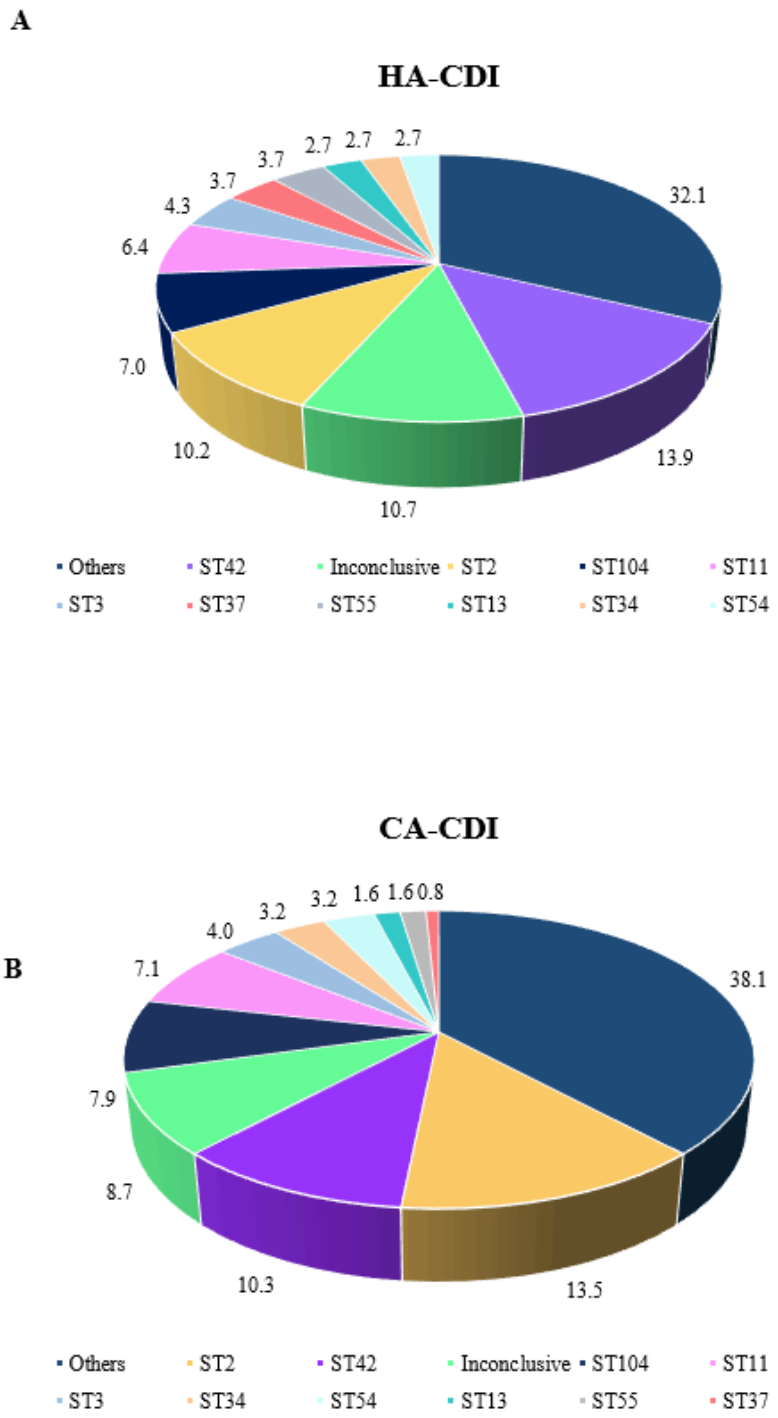

Figure 1

Distribution of *C. difficile* sequence types (STs) among study isolates. *C. difficile* isolates were typed by MLST. The figure presents the distribution of STs among *C. difficile* isolates that were recovered from stool samples of patients with (a) HA-CDI (n=187), (b) CA-CDI (n=126).

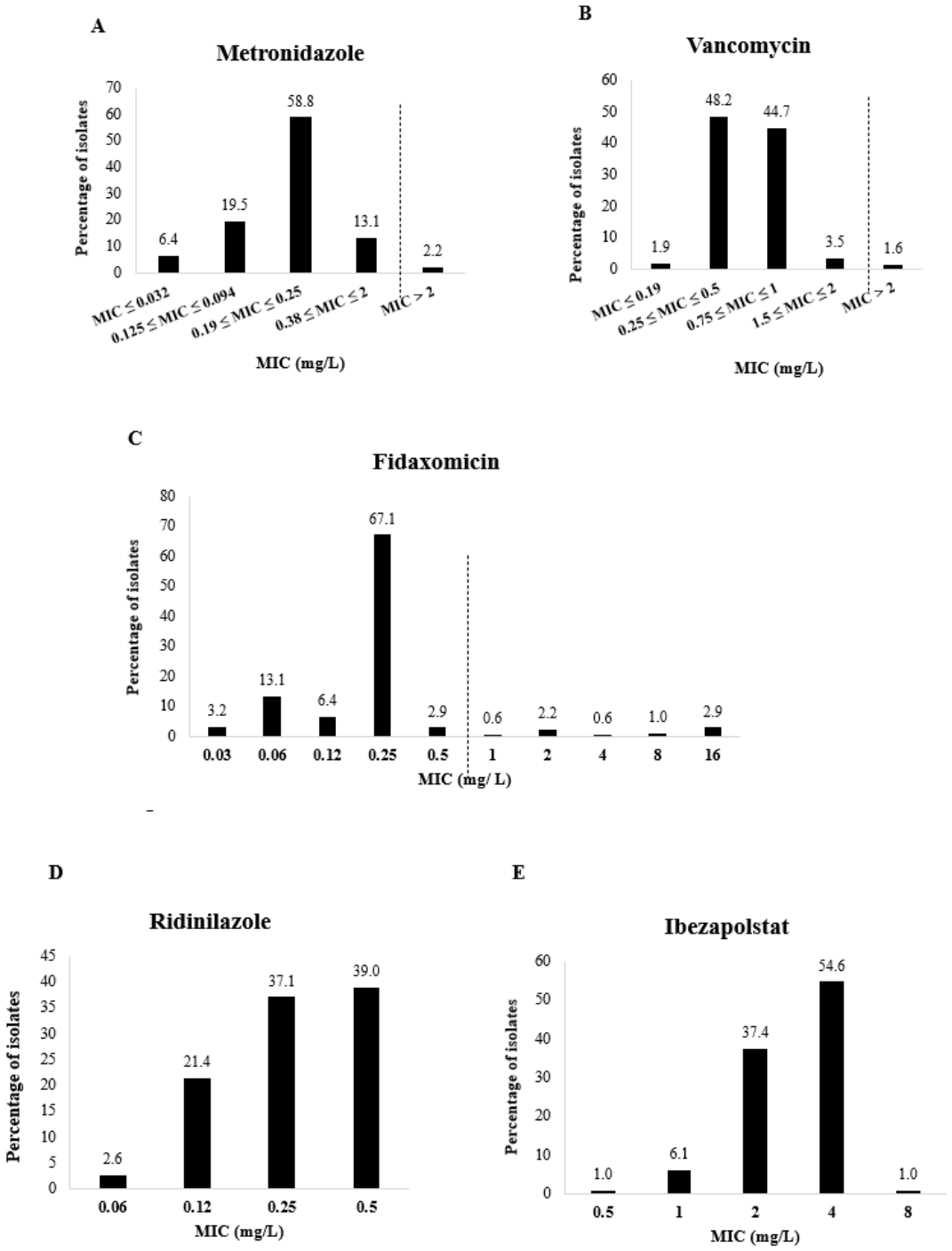

Figure 2

Distribution of MIC values of study isolates to different antibiotics. *C. difficile* susceptibility to antibiotics was determined by the E test or by agar dilution methods. The figure presents the distribution of MIC values of all study isolates to the following antibiotics (a) metronidazole, (b) vancomycin, (c) fidaxomicin, (d) ridinilazol and (e) ibezpolstat. The dashed line represent the breakpoints for resistance determination (MIC >2 mg/l for MTZ and VAN, and MIC >0.5 mg/L for FDX).

Supplementary Files

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- SupplementalTable1.docx