

## Metronidazole Resistance in *Clostridium difficile* Is Heterogeneous<sup>▽</sup>

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**At our institution, the prevalence of clinical isolates of *Clostridium difficile* with resistance to metronidazole is 6.3%. We observed that initial metronidazole MICs of 16 to 64 mg/liter against toxigenic, primary fresh *C. difficile* isolates, as determined by agar dilution, decreased to 0.125 mg/liter after the isolates were thawed. In this study, we examined the possibility of heterogeneous or inducible resistance. Totals of 14 metronidazole-resistant and 10 metronidazole-susceptible clinical isolates of toxigenic *C. difficile* were studied. The isolates were investigated for the presence of *nim* genes by PCR. After the isolates were thawed, susceptibility testing was done by agar dilution, by disc diffusion using a 5- $\mu$ g metronidazole disc, and by the Etest method. An experiment for determining the effect of prolonged exposure to metronidazole was applied to all resistant isolates and to susceptible control strains. None of the isolates presented the *nim* genes. All initially metronidazole-resistant *C. difficile* isolates became susceptible after thawing; however, they presented slow-growing subpopulations within the inhibition zones of both the disk and the Etest strip. All metronidazole-susceptible isolates remained homogeneously susceptible by both methods. After prolonged exposure in vitro to metronidazole, no zone of inhibition was found around the 5- $\mu$ g disk in any of the metronidazole-resistant isolates, and the MICs as determined by the Etest method ranged from 0.125 to >256 mg/liter, with colonies growing inside the inhibition zone. Our results indicate that (i) resistance to metronidazole was not due to the presence of *nim* genes, (ii) resistance to metronidazole in toxigenic *C. difficile* isolates is heterogeneous, and (iii) prolonged exposure to metronidazole can select for in vitro resistance. We recommend routine performance of the disk diffusion method (5- $\mu$ g metronidazole disk) with primary fresh *C. difficile* isolates in order to ensure that metronidazole-heteroresistant populations do not go undetected.**

*Clostridium difficile* infection (CDI) is a growing problem and the most common cause of hospital-acquired infectious diarrhea (3, 11, 17, 18). The first-line drugs for the therapy of CDI are metronidazole and vancomycin, although metronidazole is preferred to vancomycin because of its lower cost and because, purportedly, it does not increase the appearance of vancomycin-resistant enterococci. Most CDIs respond to either drug; however, both drugs have been associated with variable relapse rates ranging from 7 to 20% (1, 20, 23). Reports of *C. difficile* strains showing resistance to metronidazole are highly unusual in the literature, with only anecdotal descriptions (2, 5, 6, 16, 19, 21, 29). However, in a previous study, we reported that 6.3% of 415 *C. difficile* isolates, recovered over a period of 8 years in our institution, were resistant to metronidazole (22).

Recently, we observed that initial metronidazole MICs of 16 to 64 mg/liter against primary fresh, toxigenic *C. difficile* isolates, as determined by the standard agar dilution technique, decreased to 0.125 mg/liter either after thawing of the isolates or after serial passages onto brucella blood agar. This characteristic has also been described for other anaerobic bacterial species, such as *Bacteroides* spp. (10), and has been associated with the presence of *nim* genes.

To determine whether metronidazole resistance may manifest through heteroresistance that is selected via in vitro and,

possibly, in vivo exposure to the drug, we conducted a series of in vitro investigations. These included testing of initially resistant and susceptible isolates after both a freeze-thaw cycle and a serial passage on metronidazole-free medium, comparison of test results by use of a metronidazole disk and Etest strips, reselection of resistant subpopulations through in vitro exposure to metronidazole, and testing of isolates for the presence of *nim* genes.

### MATERIALS AND METHODS

**Bacterial isolates and patients.** A total of 14 unique toxigenic human clinical isolates of *C. difficile* resistant to metronidazole, as determined by the agar dilution method (MIC > 8 mg/liter), out of the original 26 isolates described in our previous study (22) were viable upon subculture from frozen stocks and studied. The strains were isolated from nonrelated patients admitted to different wards in different years, and no clonal dissemination of the isolates was observed, as determined by ribotyping.

In addition, we studied 10 clinical isolates fully susceptible to metronidazole (MICs < 0.125 mg/liter) and two susceptible control strains, *C. difficile* ATCC 9689 and *Bacteroides fragilis* NCTC 9343.

The isolates were identified by a combination of conventional phenotypic tests (15) and molecular methods by amplification of a 270-bp fragment of the 16S rRNA gene (12). The presence of *C. difficile* toxins A and B was determined directly from the isolates grown on brucella blood agar by an enzyme immunoassay and demonstrating a specific cytopathic effect on MRC-5 cells (25, 27).

All isolates of *C. difficile* had been stored frozen in skim milk without metronidazole at  $-70^{\circ}\text{C}$  after primary isolation and previous determination of susceptibility to metronidazole by the standard agar dilution method (7).

In this study, we define a fresh isolate as the primary isolate recovered from the feces of a patient with CDI in metronidazole-free solid medium (cycloserine-cefoxitin-fructose agar).

The clinical records of the 14 patients with heteroresistant isolates as well as those of the 10 patients with susceptible isolates were reviewed.

In addition, we also studied a total of 128 consecutive primary fresh clinical

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TABLE 1. Variations in susceptibility of clinical isolates of *Clostridium difficile* to metronidazole

Isolate	Ribotype	MIC (mg/liter) determined by agar dilution			Zone size ( $\varnothing^a$ ) after thawing	MIC (mg/liter) determined by Etest after thawing
		Original	After thawing	After serial passages		
1	R5	64	8	0.5	0	2
2	R14	64	8	0.5	0	1.5
3	R2	32	2	0.25	0	1
4	R2	32	2	0.25	10	0.5
5	R3	32	1	0.125	10	0.5
6	R4	32	1	0.125	12	0.5
7	R6	32	0.5	0.125	14	0.38
8	R1	16	0.25	0.125	14	0.125
9	R1	16	0.25	0.125	18	0.125
10	R1	16	0.125	0.125	18	0.125
11	R1	16	0.125	0.125	20	0.125
12	R1	16	0.125	0.125	20	0.125
13	R1	16	0.125	0.125	20	0.125
14	R1	16	0.125	0.125	22	0.125

<sup>a</sup>  $\varnothing$ , diameter in millimeters. In all cases, a heterogeneous subpopulation of small colonies was observed growing inside the inhibition zone of the disk.

isolates of toxigenic *C. difficile* recovered in our laboratory over a period of 2 months (January and February 2008) and reviewed the clinical records of the patients.

**PCR ribotyping.** The 14 resistant isolates were ribotyped as described previously (4).

**Determination of metronidazole susceptibility.** As indicated above, initial determination of the metronidazole susceptibility of each isolate was performed by the standard agar dilution technique (7). The CLSI criteria were used for the definition of resistance to metronidazole: MICs below the therapeutic breakpoint of 16 mg/liter were considered susceptible (CLSI). After the determination of the MIC, the isolates were stored frozen at  $-70^\circ\text{C}$  in skim milk without metronidazole.

After the isolates were thawed, susceptibility was redetermined by agar dilution as well as by the disk diffusion method on brucella blood agar with a 5- $\mu\text{g}$  metronidazole disk. Isolates with zone diameters of 28 to 30 mm or larger were considered susceptible (10). In addition, metronidazole MICs were determined by the Etest method (AB Biodisk, Solna, Sweden) on brucella blood agar plates according to the manufacturer's instructions. The same inoculum (1 McFarland standard) in brain heart infusion was used for both methods. Readings were performed with an amplification lens after incubation for 48 h at  $37^\circ\text{C}$  under anaerobic conditions. In addition, a second reading of the Etest strip was performed after 5 days of incubation. Gram staining and malachite green staining of the isolates growing inside the inhibition zone of the disk were performed at 48 h.

Metronidazole MICs against the 128 primary fresh clinical isolates of *C. difficile* were also determined by the disk diffusion and Etest methods as follows: several colonies obtained from the primary culture of feces onto cefoxitin- fructose agar medium were streaked onto a brucella blood agar plate and incubated for 48 h at  $37^\circ\text{C}$  under anaerobic conditions. The disk diffusion and Etest methods were used with the colonies grown on these plates as described above.

**Selection of metronidazole-resistant subpopulations by disk diffusion.** All 14 primary resistant isolates, including the 10 clinical isolates fully susceptible to metronidazole as well as *C. difficile* ATCC 9689 and *B. fragilis* NCTC 9343, were tested in order to detect whether metronidazole-resistant subpopulations existed. A disk diffusion method for selection or induction of metronidazole resistance was used as described by Gal and Brazier (10). Briefly, a sterile 30- by 30-mm membrane filter with a 0.45- $\mu\text{m}$  pore size (Millipore) was placed on the surface of a brucella blood agar plate. The total surface of the plate and the membrane was inoculated with an overnight culture of the different isolates of *C. difficile* under testing and the two control strains by means of a swab. A 5- $\mu\text{g}$  metronidazole disk was then placed in the middle of the membrane, and the plate was incubated anaerobically for a total of 14 days. Every 48 h (weekends included), the membrane filter was transferred to a fresh brucella blood agar plate, and the metronidazole disk was removed and replaced with a new one. Under these circumstances, the continuous presence of active antibiotic was ensured.

At weekly intervals, any slow-growing colonies that appeared within the initial zone of susceptibility were subcultured onto brucella blood agar without metronidazole and checked for identity and retested by the Etest method to determine whether the MIC had changed from its original value.

To verify that any increased resistance to metronidazole was not due to subculturing procedures, a series of subcultures of the initial isolates was performed using brucella blood agar plates without metronidazole for a period of 14 days, with the MIC being determined after this time.

**Studies on the stability of metronidazole-resistant isolates.** The stability of metronidazole resistance of primary fresh isolates was evaluated by performing seven serial passages over 14 days on brucella blood agar plates without antibiotic and by redetermining the MIC. The stability of resistance was also determined after the experiment for selection of metronidazole-resistant isolates by disk diffusion.

We considered the resistance stable when the MIC of metronidazole against *C. difficile* after the passages was maintained (within  $\pm 1$  dilution). We considered the resistance unstable when, with the same procedure, resistant strains became susceptible.

**Detection of *nim* genes.** The presence of *nim* genes (*nimA*, *nimB*, *nimC*, *nimD*, and *nimE*) was determined by PCR amplification followed by sequence analysis of all *C. difficile* isolates studied (14 heteroresistant and 10 fully susceptible), as previously described (28).

The positive control strains containing *nim* genes included *B. fragilis* 638R (pIP417) (*nimA*), *B. fragilis* BF8 (*nimB*), *B. fragilis* 638R (pIP419) (*nimC*), *B. fragilis* 638R (pIP421) (*nimD*), and *B. fragilis* ARU6881 (*nimE*) (26).

*B. fragilis* NCTC 9343 and *C. difficile* ATCC 9689 were included as *nim*-negative controls.

## RESULTS

**PCR ribotyping.** The 14 metronidazole-resistant isolates were grouped into seven different ribotypes (Tables 1 and 2) as follows: R1 (seven isolates), R2 (two isolates), and R3, R4, R5, R6, and R14 (one isolate each).

Among the 14 strains studied, none of them belonged to ribotype 027/NAP1.

**Determination of metronidazole MICs by agar dilution and clinical data.** The metronidazole MICs against the 14 primary *C. difficile* fresh isolates tested were 64 mg/liter (2 isolates), 32 mg/liter (5 isolates), and 16 mg/liter (7 isolates). The MICs against the 10 susceptible isolates were in all cases  $<0.125$  mg/liter (Table 1).

None of the patients with either resistant or susceptible *C. difficile* isolates had received metronidazole in the previous 6 months before the episode of CDI. Among the 14 patients from which heteroresistant *C. difficile* strains were isolated, 2 received treatment with vancomycin, and another 2 patients were treated for less than 5 days with metronidazole. The

TABLE 2. Induction of resistance of heteroresistant *C. difficile* isolates

Isolate	Ribotype	Zone size (mm) after induction	MIC (mg/liter) determined by Etest after induction <sup>a</sup>
1	R5	0	2 (256)
2	R14	0	1.5 (128)
3	R2	0	1 (64)
4	R2	0	0.5 (32)
5	R3	0	0.5 (32)
6	R4	0	0.5 (32)
7	R6	0	0.38 (16)
8	R1	0	0.125 (16)
9	R1	0	0.125 (16)
10	R1	0	0.125 (16)
11	R1	0	0.125 (16)
12	R1	0	0.125 (8)
13	R1	0	0.125 (8)
14	R1	0	0.125 (8)

<sup>a</sup> In each case, a resistant subpopulation (whose MICs are given in parentheses) was observed growing inside the inhibition zone of the Etest strip.

remaining 10 patients with metronidazole-resistant isolates completed a treatment of 7 days with metronidazole and had a recurrent episode of CDI after the therapy. None of the 10 patients with metronidazole-susceptible isolates had a recurrent episode of CDI after metronidazole treatment.

**Determination of susceptibility to metronidazole by disk diffusion and by the Etest method.** (i) After thawing, all initially resistant isolates showed either a reduced zone of susceptibility (10- to 22-mm diameter) or no zone of inhibition at all on a 5- $\mu$ g metronidazole disk (Fig. 1). Metronidazole MICs ranged from 0.125 to 2 mg/liter at 48 h by the Etest method (Table 1).

Among the 10 susceptible isolates, the diameters of inhibition of the metronidazole disk ranged from 36 mm to 50 mm and the MICs were <0.125 mg/liter in all cases.

(ii) Among the 128 consecutive primary fresh isolates of *C. difficile*, 93 isolates were fully susceptible and 35 presented heteroresistance. The disk diffusion method detected the 35

heteroresistant isolates; however, the Etest method detected heteroresistance in only 16 isolates (Fig. 1). None of the patients with heteroresistant isolates (35 patients) had received metronidazole in the previous 6 months.

**Selection of metronidazole-resistant subpopulations by disk diffusion.** As indicated above, the 14 resistant isolates showed no zone of inhibition (3 isolates) or an inhibition zone ranging from 10 to 22 mm. However, the selection experiment showed that the diameter of the inhibition zone around the metronidazole disk became successively smaller over time. At 48 h, small colonies began to appear inside the inhibition zone. Gram staining and malachite green staining of the isolates growing inside the disk inhibition zone at 48 h of incubation did not reveal sporulation.

The slow-growing colonies from around the metronidazole disk were subcultured after incubation for 2 weeks, and the MICs were redetermined by the Etest method. Again, a heterogeneous population was observed in all isolates: a subpopulation inhibited with the same MICs as before the selection (MICs ranging from 0.125 to 2 mg/liter) and another subpopulation of smaller colonies growing within the ellipse area of the Etest strip after incubation for 48 h. These subpopulations showed MICs ranging from 0.125 to >256 mg/liter (Table 2).

We redetermined the MICs by the Etest method after serial passages onto metronidazole-free brucella blood agar. Starting from the first passage, we observed a progressive decrease in the number of colonies growing inside the ellipse of inhibition of the Etest strip. After successive passages, the heterogeneous population began to disappear and the heteroresistant isolates became susceptible.

No decreased inhibition zones, increased MICs, or slow-growing resistant colonies were found when the same experiment was performed using either the *C. difficile* ATCC 9689 control strain, the *B. fragilis* NCTC 9343 strain, or the 10 metronidazole-susceptible clinical isolates.

**Instability of resistance to metronidazole.** After the isolates were thawed, the initial metronidazole MICs against the 14 resistant isolates showed decreases from 8- to more than 64-fold, as determined by agar dilution. After serial passages onto

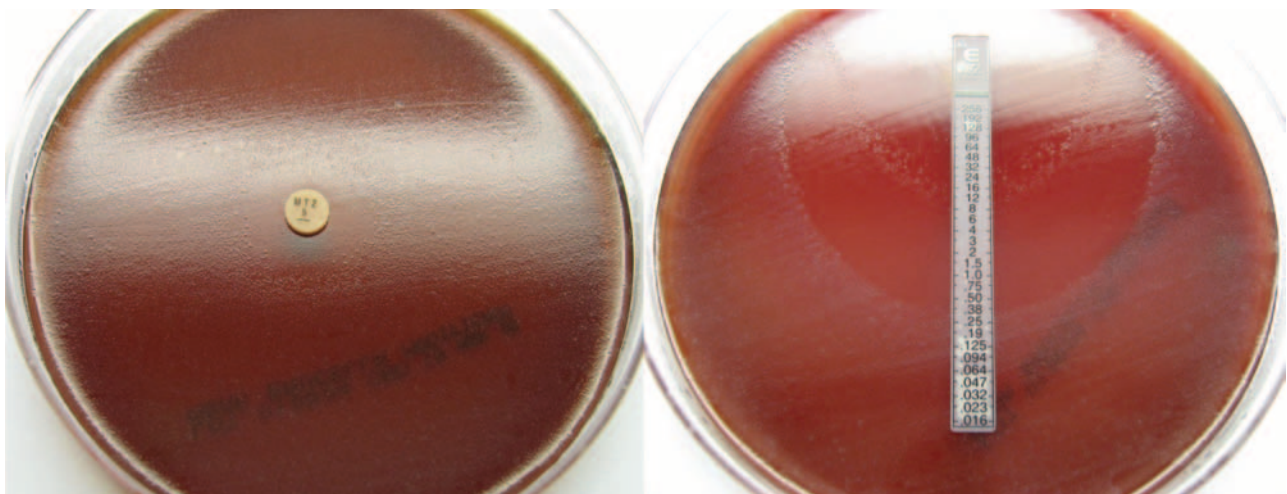


FIG. 1. Detection of a heteroresistant *C. difficile* isolate by disk diffusion and by the Etest method.

metronidazole-free brucella blood agar, all initially resistant isolates became susceptible (MICs from 0.125 to 8 mg/liter) (Table 1). The MICs against the 10 susceptible strains remained unchanged either after thawing or after serial passages onto metronidazole-free medium.

**Detection of *nim* genes.** None of the clinical *C. difficile* isolates (heteroresistant or susceptible to metronidazole) carried *nim* genes (*nimA*, *nimB*, *nimC*, *nimD*, and *nimE*). All the *nim*-positive controls were positive by PCR and sequencing. The *nim*-negative controls were negative by PCR.

## DISCUSSION

In this study, we have documented that the metronidazole resistance of toxigenic clinical *C. difficile* isolates is heterogeneous and inducible and is not due to the presence of *nim* genes. To our knowledge, resistance due to *nim* genes in *C. difficile* has not been described to date. In other anaerobic species, such as *Bacteroides* spp., resistance to metronidazole is, in general, due to the presence of *nim* genes; however, other alternative metronidazole resistance mechanisms have been suggested, such as reduced uptake of metronidazole, reduced nitroreductase activity, or decreased pyruvate-ferredoxin oxidoreductase activity (8, 10). Among the resistant isolates, we observed two subpopulations (a majority subpopulation that was susceptible to metronidazole and a minority subpopulation that was resistant and inducible), and this characteristic was observed in different unrelated *C. difficile* isolates, as determined by ribotyping. We have also demonstrated that resistance to metronidazole in *C. difficile* is unstable. First, the initial MICs determined for fresh isolates decreased after the isolates were thawed, and second, the observed increases in MICs against the isolates obtained after induction in the presence of subinhibitory concentrations of metronidazole were followed by reversion to susceptibility levels in the absence of metronidazole. This phenomenon might be similar to the heterogeneous resistance that has been described for *Bacteroides* spp. and *Helicobacter pylori* (9, 10, 13, 14). As in *Bacteroides* spp., heteroresistant *C. difficile* isolates for which the MICs of the majority subpopulation were below the breakpoint also presented slow-growing subpopulations with higher metronidazole MICs, detected only in primary fresh cultures by disk diffusion or by the Etest method after prolonged incubation. Although the Etest method is much less laborious and is easier to perform than the agar dilution method, especially for routine purposes, in our study this method did not detect all the isolates showing heteroresistance either in primary fresh isolates or after thawing. In addition, it has been described that metronidazole MICs for *C. difficile* determined by the Etest method were lower than those obtained by agar dilution (24).

The metronidazole heteroresistance of *C. difficile* isolates is an unstable mechanism that goes undetected if metronidazole MICs are determined by the CLSI standard agar dilution method after the isolates are thawed. Most clinical laboratories do not perform metronidazole susceptibility testing of primary fresh isolates, and isolates are tested after a freeze-thaw cycle and after a number of passages in vitro. The CLSI reference agar dilution method for anaerobes (7) recommends that frozen isolates be subcultured by at least two serial transfers on supplemented blood agar without antibiotic prior to testing.

Under these circumstances, the number of colonies that can be recovered from the frozen state and cultured on agar plates is normally  $<1 \times 10^3$ , so the number of colonies obtained on agar plates may be too small for regular transmission of heterogeneous resistance (13). After drug-free passages, the slow-growing resistant subpopulation decreases and is replaced by a metronidazole-susceptible subpopulation with a higher growth rate. For this reason, and in order to avoid the overgrowth of susceptible colonies, we recommend that susceptibility to metronidazole be determined by disk diffusion, using several colonies obtained from the first passage of the primary culture (see Materials and Methods). This method allows the detection of heteroresistant isolates.

Our results also showed that the initially heteroresistant *C. difficile* isolates, which became apparently susceptible after passages onto drug-free medium, showed either no zone or visible resistant colonies inside the inhibition zone of the metronidazole disk after exposure to metronidazole in vitro. In contrast, no colonies were observed inside the inhibition zone when susceptible isolates were tested. Moreover, heteroresistance was easily detected in primary fresh clinical isolates of *C. difficile* by the disk diffusion method. The high sensitivity of the disk diffusion method for the detection of resistant *C. difficile* isolates observed in our study in comparison with the sensitivity of the Etest method is probably due to the fact that subinhibitory concentrations of metronidazole inside the inhibition zone of the disk facilitate the expression of resistance of *C. difficile* isolates and the visualization of a heteroresistant population. As indicated above, this observation has been previously described for *Bacteroides* spp. (10). As a consequence, in clinical laboratories where the determination of susceptibility by the agar dilution method is labor-intensive and costly, we recommend routine performance of the disk diffusion method (5- $\mu$ g metronidazole disk) with primary *C. difficile* isolates in order to ensure that metronidazole-heteroresistant populations do not go undetected. This recommendation is very important since our preliminary clinical data demonstrated that there was a correlation between heteroresistance and poor clinical outcome.

In conclusion, if heteroresistant *C. difficile* strains can contribute to therapeutic failure in specific clinical situations, as described in our study, continued surveillance is needed. The disk diffusion method is an easy and rapid method which allows the detection of heteroresistance.

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