IN VITRO SUSCEPTIBILITY TO METRONIDAZOLE IN DIFFERENT *C. DIFFICILE* PCR-RIBOTYPES

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1. Introduction

Metronidazole remains the drug of choice in the treatment of mild to moderate *C. difficile* infection cases, even if cases of treatment failure have been recently reported.

Reduced susceptibility and heteroresistance have been observed in some *C. difficile* isolates, but it is unclear if *C. difficile* strains *in vitro* susceptible to metronidazole can show heterogenic MICs under antibiotic pressure that would help understand the behaviour of strains that do not respond to treatment *in vivo*.

In this study, three different methods were used to determine the susceptibility to metronidazole of 81 *C. difficile* strains, belonging to prevalent or emergent PCR-ribotypes, and to investigate the heterogeneity of MIC values in *C. difficile* isolates, before and after exposure to sub-inhibitory concentrations of the antibiotic.

2. Materials and Methods

Eighty one *C. difficile* clinical isolates were selected to this study. Twenty three strains were characterized at the Istituto Superiore di Sanità (ISS) between 2006 and 2011. Fifty strains were collected in 2005 as part of a European surveillance study (CMI. 2007.13: 048–57). The remaining strains were kindly provided by E. Bouza (Spain), E. Kuijper (the Netherlands), N. Minton (England) and M. Popoff (France). One strain PCR-ribotype 001, previously characterized as reduced susceptible to metronidazole, was provided by M. Wilcox (England).

Etest was carried out according to the manufacturer's instructions (AB Biodisk) in Brucella agar (BA) plates. Agar dilution method (ADM) and agar incorporation method (AIM) were performed as recommended by the CLSI (CLSI, 2007) and by Freeman et al. (JAC. 2005. 56: 988–9), respectively. The pre-culture extension and the inoculum differed in ADM and AIM. Briefly, to evaluate the MICs by ADM the strains were suspended in Brucella broth and multipoint inoculated onto BA plates. AIM was carried out by inoculating *C. difficile* strains cultured in Schaedlers anaerobic broth for 48h onto Wilkins-Chalgren agar plates.

Epidemiological cut-off (ECOFF) for reduced susceptibility to metronidazole was defined as MIC > 2 mg/L, according to The European Committee on Antimicrobial Susceptibility Testing (EUCAST) (Table version 2.0 valid from 2012-01-01).

Twenty strains, representative of the different PCR-ribotypes analysed in this study and fully susceptible to metronidazole, were exposed to sub-inhibitory concentrations of metronidazole and after 24h their MICs were evaluated by Etest. The strains showing MIC increase of at least two-fold by Etest were also analysed by ADM and AIM. Twenty single colonies were randomly isolated from each strain, before and after antibiotic exposure, and their MICs were evaluated by both ADM and AIM.

3. Results

3.1. Susceptibility testing

Overall, the MIC values determined by agar incorporation method (AIM) were higher compared with those obtained by agar dilution method (ADM), whereas the lowest MICs were obtained using Etest. The of MIC values of the 81 strains are reported in Table 1.
The highest MIC values were observed among the strains belonging to PCR-ribotypes 001 and 010 by all three methods.

Only one isolate PCR-ribotype 010, showed a high MIC value (≥ 16 mg/L) by all methods.
Two strains belonging to PCR-ribotype 010 showed reduced susceptibility to metronidazole by ADM and AIM. Three other strains, one belonging to PCR-ribotype 001 and two belonging to PCR-ribotype 010 showed reduced susceptibility only by AIM. Therefore, the categorization of *C. difficile* strains as susceptible or with reduced susceptibility may differ according to the method used (Figure 1).

All the other strains investigated were fully susceptible to metronidazole by all three methods.

Table 1. Range of MICs of the 81 *C. difficile* clinical isolates analysed in this study by three different methods

PCR– ribotype (n. of strains)	MIC (mg/L)		
	Etest	Agar dilution method	Agar incorporation method
027 (8)	0.032 – 0.19	0.125 – 0.5	0.125 – 0.5
078 (12)	0.047 – 0.25	0.125– 0.25	0.125
012 (10)	0.032 – 0.125	0.064 – 0.25	0.125 – 0.25
001 (11)	0.047 – 0.5	0.064 – 1	0.125 – 4
018 (10)	0.032 – 0.25	0.125 – 0.5	0.125 – 1
010 (10)	0.032 – 24	0.125 – 32	0.125 – 16
014 (5)	0.064 – 0.125	0.125	0.25
020 (5)	0.064 – 0.125	0.125	0.125 – 0.25
126 (10)	0.047 – 0.125	0.125 – 0.25	0.125

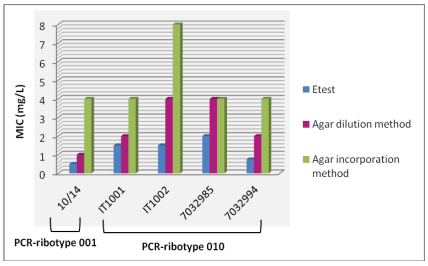

Figure 1. MICs to metronidazole of the *C. difficile* strains differently categorized as susceptible or with reduced susceptibility according to the method (ECOFF > 2 mg/L)

3.2. *"in vitro"* exposure to metronidazole

Following the antibiotic exposure, three strains belonging to PCR-ribotypes 001 and one strain PCR-ribotype 010 showed MIC increase of at least two-fold by Etest and ADM, but only one of them showed MIC increase by AIM (Table 2).

The twenty selected colonies isolated from each strain showed heterogeneity in their MIC values, before and after antibiotic exposure. The MICs of the colonies increased by ADM after *in vitro* exposure to metronidazole, whereas less variations, before and after antibiotic exposure, were observed by AIM (Figure 2).

Table 2. MICs of *C. difficile* isolates belonging to PCR-ribotypes 001 and 010, before and after *in vitro* exposure to metronidazole

Strains	PCR-ribotype	MIC (mg/L)					
		Before antibiotic exposure			After antibiotic exposure		
		Etest	Agar dilution method	Agar incorporation method	Etest	Agar dilution method	Agar incorporation method
DII15	001	0.094	0.5	0.5	**0.75**	**1**	**1**
FIII10	001	0.047	0.064	0.25	**0.25**	**0.125**	**0.25**
10/14	001	0.5	1	4	**1**	**2**	**4**
7032985	010	2	4	4	**4**	**8**	**4**

ECOFF > 2 mg/L

4. Conclusions

- Among the nine PCR-ribotypes analysed in this study, reduced susceptibility was observed only in *C. difficile* isolates belonging to PCR-ribotypes 001 or 010.

- The overall data reported in this study suggest AIM as the method of choice to detect strains with reduced susceptibility to metronidazole

- The colonies analysed in this study showed heterogenic MICs before and after antibiotic exposure, but higher MIC values were reached after antibiotic exposure, suggesting that antibiotic pressure could have a relevant role in selecting *C. difficile* sub-populations with reduced susceptibility to metronidazole.

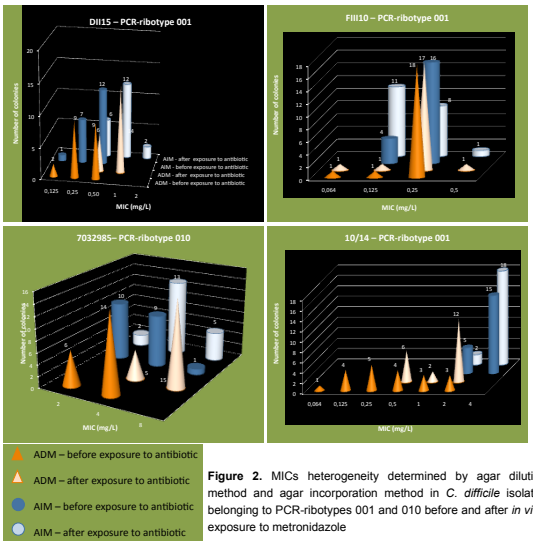

Figure 2. MICs heterogeneity determined by agar dilution method and agar incorporation method in *C. difficile* isolates belonging to PCR-ribotypes 001 and 010 before and after *in vitro* exposure to metronidazole

Aknowledgements

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