MOLECULAR EPIDEMIOLOGY AND ANTIMICROBIAL RESISTANCE OF *CLOSTRIDIUM DIFFICILE* ISOLATES IN BELGIUM

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Introduction

Vancomycine and metronidazole are the drugs of choice for the treatment of *Clostridium difficile* infections. Recent communications on reduced susceptibility to metronidazole and the lack of data on antimicrobial resistance among the different ribotypes in Belgium was the aim of this study. Since 2006, we typed more than 3000 *C. difficile* strains in our national reference center. Over a one year period from July 2008 until June 2009 we typed 909 strains from all over the country and from our University Hospital, using PCR-ribotyping and DNA fragments analysis by capillary electrophoresis.

Materials and methods

Strains: Sporulated strains were preserved in a semi-solid thioglycolate medium at room temperature. Control strains for each of *C. difficile* ribotypes 027, 014, 020, 002, 078, 015 and 001 were obtained from the Department of Medical Microbiology, University Medical Center, Leiden. Other ribotypes were given internal numbers.

Cultures: on CCFA+T (cycloserine cefotaxime fructose agar additionned with sodiumtaurocholate)

Ribotyping : DNA extraction was performed using Chelex 100 at 5%. RT-PCR was performed on a LightCycler 480 (Roche), electrophorograms were read using a Genescan.

Minimal Inhibitory Concentrations (MIC) were performed with MIC teststrips from Liofilchem (Elitech) for moxifloxacin, vancomycin and metronidazole and with E-tests from AB Biodisk (bioMérieux) for moxifloxacin. A 2 MacFarland suspension from a 24 hours culture was inoculated on a Columbia blood plate (bioMérieux) and incubated with the respective antibiotic strip in anaerobic conditions for 48 hours and read.

Results

Over a 12 month period (July 2008-June 2009), 909 strains of *C. difficile were* ribotyped in the national reference laboratory, in Belgium. Two hundred and two strains were from our St Luc University Hospital. The distribution and frequency of the different ribotypes was almost the same in our hospital as in Belgium.(table 1, table 2)

We studied a selection of 96 strains among the eight most frequent ribotypes in Belgium for their susceptibility to moxifloxacin, vancomycin and metronidazole. We calculated MIC 50 and MIC 90 (table 3) for each antibiotic and ribotype. We could show a clear reduced susceptibility to metronidazole for the NAP/027 strains compared to other ribotypes (fig.1). For vancomycin there was no significant difference between the tested ribotypes (fig. 2).

All but one NAP1/027 strains were resistant to moxifloxacin. We detected also other ribotypes that were resistant for moxifloxacin (table 4). One of them had no deletion in the tcdC gene nor a binary toxin .

We compared the E tests for moxifloxacin from AB Biodisk (bioMérieux) and MIC striptests from Liofilchem (Lucron,Elitech) on 96 strains. Between MIC's from 0.5 to 1.5 µg/ml, the Liofilchem strip gave systematically one dilution lower (fig.3). The strips are rigid and lecture was as easy as for traditional E-tests

Fig.3 Comparason of moxifloxacine E-tests,AB Biodisk (bioMérieux) and MIC striptests Liofilchem (Lucron, Elitech)

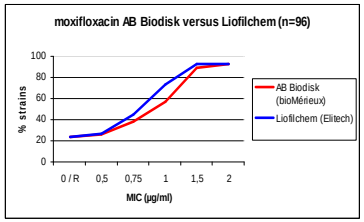


Table 1 Most frequent ribotypes found in Belgium (Jul 2008-Jun 2009, total nr of strains n=909)

Number strains	%	Ribotype Brussels	Ribotype Brazier
197	22	027	027
80	9	16	014
52	6	16a	020
36	4	32	002
35	4	46	?
34	4	3	078
30	3	23	015
27	3	23b	001

Table 2 Most frequent ribotypes found in Cliniques Universitaires St Luc Brussels (Jul 2008-Jun 2009, total nr of strains n=203)

Number strains	%	Ribotype Brussels	Ribotype Brazier
23	11	16	014
14	7	16a	020
13	6	32	002
12	6	027	027
10	5	46	?
9	4	3	078
7	3	9	?
7	3	23	015

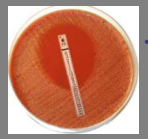


Table 3 MIC 50 and MIC 90 for metronidazole, vancomycin and MIC for moxifloxacin

metronidazole (µg/ml)

Ribotype Brussels	027	16	23	23b	3	32	46
Ribotype Brazier	027	014	015	001	078	002	?
number of strains	n=20	n=20	n=12	n=8	n=12	n=12	n=12
MIC 50	1	0,19	0,19	0,125	0,19	0,25	0,19
MIC 90	1,5	0,38	0,25	0,5	0,25	0,75	0,25
Variation	0,094 - 1,500	0,032 - 0,750	0,094 - 0,380	0,125 - 0,500	0,094 - 0,500	0,064 - 1,000	0,094 - 0,380

vancomycin (µg/ml)

Ribotype Brussels	027	16	23	23b	3	32	46
Ribotype Brazier	027	014	015	001	078	002	?
number of strains	n=20	n=20	n=12	n=8	n=12	n=12	n=12
MIC 50	0,25	0,25	0,25	0,25	0,25	0,38	0,38
MIC 90	0,38	0,38	0,38	0,25	0,5	0,5	0,5
Variation	0,190 - 0,380	0,190 - 0,500	0,190 - 0,500	0,190 - 0,750	0,190 - 0,500	0,250 - 0,500	0,190 - 0,500

moxifloxacin (µg/ml)

Ribotype Brussels	027	16	23	23b	3	32	46
Ribotype Brazier	027	014	015	001	078	002	?
number of strains	n=20	n=20	n=12	n=8	n=12	n=12	n=12
MIC 0 / R	19	0	2	0	3	0	0
MIC 0,5	0	0	1	1	1	1	0
MIC 0,75	1	2	5	4	1	3	1
MIC 1	0	7	4	2	3	5	8
MIC 1,5	0	11	0	1	2	3	3

Fig.1 Cumulative percentages of strains (n=96) versus MIC for metronidazole

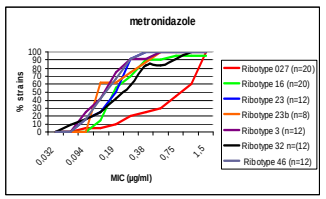


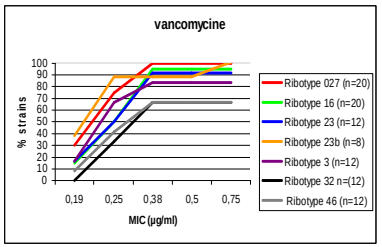
Table 4 Comparason of MIC distribution at St Luc versus Belgium

moxifloxacin

MIC	St-Luc Brussels	%	Belgium	%
0 / R	10	10	14	15
0,5	2	2	2	2
0,75	8	8	11	11
1	19	20	10	10
1,5	9	9	11	11

Ribotype Brussels	027	23	3	116a	1
Ribotype Brazier	027	015	078	?	?
number of strains	n=20	n=12	n=12	n=2	n=2
resistant strains	n=19	n=3	n=3	n=1	n=2
deletion tcdC gene	pos	neg	pos	?	?
binary toxin	pos	neg	pos	?	?

Fig.2 Cumulative percentages of strains (n=96) versus MIC for vancomycin



Discussion

In the NAP1/027 strains that we analyzed we found a significant reduced susceptibility to metronidazole compared to other ribotypes. All but one NAP1/027 were resistant to moxifloxacin. We detected moxifloxacin resistance in other frequent ribotypes. In one of them we could not show a deletion in the tcdC gene or presence of binary toxin. New emerging ribotypes need antimicrobial surveillance and justify molecular epidemiology.

Abstract

Aim : *Clostridium difficile* associated diarrhea is a global health problem and new virulent strains are emerging. Data regarding the molecular epidemiology and antimicrobial resistance of *C. difficile* isolates in Belgium remain limited.

Materials and methods : Over a one year period from July 2008 to June 2009 our National Reference Center ribotyped 909 strains from all over the country, using PCR-ribotyping and DNA fragments analysis by capillary electrophoresis. Minimal inhibitory concentrations (MIC) of moxifloxacin, metronidazole and vancomycin were determined on 126 selected strains using E-tests susceptibility testing and MIC striptests.

Results : Among the 909 strains a total of 119 different ribotypes were identified. Ribotype 027 (21,7%), 014 (8,8%), 002 (3,9%) and 078 (3,7%) were predominant. The ribotype 027 (N=20 strains tested for AB susceptibility) showed a clear reduced susceptibility to metronidazole compared to other ribotypes: MIC 50 was 1,0 mg/L and MIC 90 1,5 mg/L as compared to MIC 50 of 0,190 mg/L and MIC 90 of 0,380 mg/L for other ribotypes (N=106). All but one 027 strains were resistant to moxifloxacin. We detected moxifloxacin resistance in 3 other ribotypes, which all showed a deletion in the tcdC gene and the presence of the binary toxin gene. These 3 ribotypes did not show a reduced susceptibility to metronidazole. The highest MIC of vancomycin was 0,75 mg/L and there was no significant difference among strains.

Conclusion New emerging ribotypes resistant to fluoroquinolones show up and justify molecular epidemiology and antimicrobial surveillance. No resistance was found towards metronidazole but a ribotype specific reduced susceptibility.

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