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Abstract: World wide increasing rates of *Clostridium difficile* infections (CDI) with severe courses and outbreaks have been reported. This change in CDI epidemiology has on one hand been related to the spread of specific PCR ribotypes (e.g. 027) and on the other hand to increased prevalence of resistant *C. difficile* strains. This single centre retrospective analysis characterized resistance against erythromycin and moxifloxacin, presence of binary toxin gene and ribotypes in 73 *C. difficile* isolates from 2008 in comparison with 23 isolates from 1990. In 1990 five different PCR ribotypes including 027 were identified. Resistance against erythromycin was detected in 3 / 23 (13 %), while 20 / 23 (87 %) of all isolates were susceptible to both, erythromycin and moxifloxacin. In contrast, in 2008 a significantly increased prevalence of resistant *C. difficile* strains was observed, with 40 / 73 (54.8 %) isolates being resistant against both antibiotics. Resistant *C. difficile* strains were mainly assigned to PCR ribotype 001. No isolates belonging to PCR ribotype 027 were identified. Our data provide evidence that increase of resistant *C. difficile* strains belonging to PCR ribotype 001 rather than the spread of *C. difficile* PCR ribotype 027 contribute to the changing epidemiology of CDI.

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Comparison of resistance against erythromycin and moxifloxacin, presence of binary toxin gene and PCR ribotypes in *Clostridium difficile* isolates from 1990 and 2008

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Abstract

World wide increasing rates of *Clostridium difficile* infections (CDI) with severe courses and outbreaks have been reported. This change in CDI epidemiology has on one hand been related to the spread of specific PCR ribotypes (e.g. 027) and on the other hand to increased prevalence of resistant *C. difficile* strains. This single centre retrospective analysis characterized resistance against erythromycin and moxifloxacin, presence of binary toxin gene and ribotypes in 73 *C. difficile* isolates from 2008 in comparison with 23 isolates from 1990. In 1990 five different PCR ribotypes including 027 were identified. Resistance against erythromycin was detected in 3 / 23 (13 %), while 20 / 23 (87 %) of all isolates were susceptible to both, erythromycin and moxifloxacin. In contrast, in 2008 a significantly increased prevalence of resistant *C. difficile* strains was observed, with 40 / 73 (54.8 %) isolates being resistant against both antibiotics. Resistant *C. difficile* strains were mainly assigned to PCR ribotype 001. No isolates belonging to PCR ribotype 027 were identified. Our data provide evidence that increase of resistant *C. difficile* strains belonging to PCR ribotype 001 rather than the spread of *C. difficile* PCR ribotype 027 contribute to the changing epidemiology of CDI.

Key words: *Clostridium difficile*, PCR ribotype, resistance, binary toxin

Clostridium difficile has long been considered a significant causative agent of nosocomially acquired diarrhoea [1]. Since 2002 increasing numbers of infections, outbreaks and severe cases of *Clostridium difficile*-infections (CDI) have been reported. In part, these infections were attributed to variant *C. difficile* strain belonging to PCR ribotype 027 [1]. This strain produces binary toxin CDT, encoded by *cdtA* and *cdtB*, and has a partial deletion of the regulatory *tcdC* gene, resulting in toxin A and B hyperproduction [2]. In addition, independent PCR ribotypes (e.g. 001, 053, 078 and 106) can cause outbreaks and severe courses of *C. difficile* infections [3], indicating that currently epidemiology of virulent *C. difficile* strains is subject to complex changes that cannot solely be attributed to the spread of one particular virulent *C. difficile* strain.

We here analyzed *C. difficile* isolates from patients hospitalized at the University Hospital Centre Hamburg-Eppendorf in 1990 (n=23) and 2008 (n=73) with clinical suspicion of CDI. Isolates from 1990 were randomly collected over the whole year. These stool samples were not tested for the presence of toxin A or B. No information about antibiotic therapies was available. *C. difficile* isolates from 2008 were consecutively collected during a three month period from patients hospitalized in different departments. 39 / 73 (53.5 %) of the stool samples were toxin positive (TechLab Quik Check, Blacksburg, USA). At the time of sample collection, 47 / 73 (64.3 %) patients received antibiotics (11 / 73 [15.1 %], no antibiotics. 15 / 73 [20.6 %], no information).

Using a published method [4] *C. difficile* isolates from 1990 were assigned to 5 different PCR ribotypes (012, n=3; 014, n=8; 027, n=2; 042, n=9; 053, n=1). PCR detected *cdtB* in 2 / 23 (8.7 %) isolates, both belonging to PCR ribotype 027. Susceptibility testing using the Etest (Biomérieux, Marcy l'Etoile, France) revealed that only 3 / 23 (13 %) *C. difficile* isolates were resistant against erythromycin, while 20 / 23 (87 %) were susceptible against moxifloxacin and erythromycin (Table 1). Both *cdtB*-positive isolates belonging to ribotype 027 were susceptible to erythromycin and moxifloxacin. In 2008 a total of 16 different PCR

ribotypes were determined. Most isolates (44 / 73; 60.3 %) were assigned to ribotype 001, 25
 isolates belonged to sporadic ribotypes (003, n=3; 010, n=1; 012, n=1; 014, n=8; 015, n=1;
 020, n=1; 023, n=1; 032, n=1; 042, n=1; 045, n=1, 046; n=1; 066, n=1; 078, n=1; 081, n=2;
 087, n=1). Four strains were not typeable, PCR ribotype 027 was not detected. *cdtB* was
 found in only 3 / 73 (4.1 %) isolates belonging to PCR ribotypes 023, 066, and 078,
 respectively. Statistical analysis revealed that *cdtB* prevalences in 1990 and 2008 did not
 differ significantly ($p=0.590$, Fisher's exact test). However, compared to *C. difficile* isolates
 from 1990, *C. difficile* isolates from 2008 exhibited increased MICs against erythromycin
 (MIC_{50} : 256 μ g/ml vs. 1.5 μ g/ml; MIC_{90} : 256 μ g/ml vs. 32 μ g/ml) and moxifloxacin (MIC_{50} :
 32 μ g/ml vs. 0.75 μ g/ml; MIC_{90} : 32 μ g/ml vs. 1 μ g/ml). 40 / 73 (54.8 %) of the isolates from
 2008 were resistant against erythromycin and moxifloxacin, an isolated resistance against
 erythromycin was detected in 4 / 73 (5.5 %) isolates and against moxifloxacin in 2 / 73 (2.7
 %) isolates (Table 1). Overall, compared with *C. difficile* strains from 1990 the proportion of
 erythromycin and moxifloxacin susceptible strains was significantly lower in *C. difficile*
 isolates from 2008 (20/23, 87 % vs. 27/73, 37 %, $p<0.001$). Importantly, 37 / 44 (84.1 %)
 isolates belonging to PCR ribotype 001, but only 2 / 29 (6.9 %) strains belonging to PCR
 ribotypes other than 001 were resistant against erythromycin and moxifloxacin. All *cdtB*-
 positive isolates from 2008 were susceptible against both agents.

Epidemiological evidence suggest that on one hand occurrence and spread of variant *C.*
difficile PCR ribotype 027 contributed to the ever increasing number of CDI cases in regions
 with epidemic outbreaks [1]. However, spread of this strain is clearly not the exclusive
 reason for the changing epidemiology and clinical presentation of CDI in general. The low
 prevalence of ribotype 027 found in our study is in line with a study investigating the
 distribution of PCR ribotypes in a European collection of 411 *C. difficile* isolates, assigning
 only 6,2% of all isolates to PCR ribotype 027 [5]. In southern Germany CDI was
 predominantly caused by *C. difficile* belonging to ribotype 001 [6]. In line with this study we

here document the importance of PCR ribotype 001 in our hospital, and it is tempting to speculate that ribotype 001 is of special importance in Germany [7].

Interestingly, we were able to demonstrate presence of *cdtB*-positive *C. difficile* ribotype 027 isolates already in a collection of 23 strains from 1990, demonstrating that ancestors of the current epidemic clone were present in virulent *C. difficile* populations almost 20 years ago. Detailed molecular comparison of these early strains with current *C. difficile* 027 isolates could provide new insights into the evolution of this particular strain, which is a prerequisite for understanding the molecular basis for its epidemic potential.

It is well accepted that antibiotic treatment is a major risk factor for *C. difficile* gut colonization, infection and the subsequent development of CDI [1]. We here document a significantly increased prevalence of erythromycin and moxifloxacin resistant *C. difficile* isolates between 1990 and 2008. It should be emphasized that it cannot be excluded that this finding results from differences between patients groups sampled in 1990 and 2008, rather than differences between *C. difficile* populations. In addition, the results could be biased by the different length of periods used for sampling. However, as especially fluoroquinolones have more recently been identified as an important risk factor for the development of CDI [8], it is reasonable to speculate that a host environment significantly exposed against fluoroquinolones favours the expansion of resistant *C. difficile* clones [9]. Indeed, several other studies lend support to this idea [10], demonstrating that significant proportions of clinical *C. difficile* isolates from different PCR ribotypes are resistant against fluoroquinolones. Strikingly, in our collection erythromycin and fluoroquinolone resistance was associated with isolates belonging to PCR ribotype 001, suggesting that the over all increase of erythromycin and moxifloxacin resistant *C. difficile* isolates is in part related to the spread of this ribotype. The multi resistant phenotype of *C. difficile* PCR ribotype 001 could, in addition to its ability to sporulate more frequently [11], explain the particular epidemic potential of this strain.

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Table 1: Distribution of erythromycin and moxifloxacin MIC values in *C. difficile* isolates from 1990 and 2008

Antimicrobial agent (year)	No. of strains for which the antimicrobial agent MIC (µg/ml) was*												Geometric mean (µg/ml)
	0,125	0,25	0,5	0,75	1	1,5	2	4	8	16	32	256	
ERY (1990)		2	1	3	2	8	4				1	2	24.75
ERY (2008)	2		2	3	7	15	1					43	151.27
MX (1990)			6	9	8								0.77
MX (2008)			14	11	7						41		18.28

* Breakpoints used to differentiate susceptible and resistant isolates were defined for ERY at ≥ 4 µg/ml (according to DIN) and for MX at ≥ 8 µg/ml (according to CLSI)

Reviewer 1

1. *This is a very short five page long paper with very few data. Only one table with original data is given (table 1) whereas the MIC of table 2 can be directly calculated from the original data in table 1.*

Table 2 was removed, geometric means were included in table 1.

2. *The authors try to demonstrate "epidemiologic changes", however, this is not possible due to limitations of the study design. Therefore, the present characterisation of these ad hoc collected *C. difficile* isolates is certainly of interest for the local situation but to very less extent to the scientific community.*

We have removed the respective sentence.

General comments

At the end of the manuscript it should clearly stated. The results of the present study are limited due to several reasons.....

In order to fit with the requirements for a brief report we saw no possibility to add the statement. However; the limitation referee 1 is referring to are mentioned in the text (page 5, line 12 ff.” It should be emphasized that it cannot be excluded that this finding results from differences between patients groups sampled in 1990 and 2008, rather than differences between *C. difficile* populations. In addition, the results could be biased by the different length of periods used for sampling.”)

1. *Abstract line 6 please change "The present study" in " The present single center retrospective analysis"*

The sentence was changed accordingly.

2. *line 14 please substitute: No isolates belonging to PCR ribotype 027 were identified "in our cohort".*

Sentence was changed.

3. *line 17 and other parts of the manuscript including page 7 line 8: The term "epidemiology" should not be used throughout the complete manuscript in this retrospective analysis with unselected samples.*

The term was, if referring to our observations, deleted.

4. *page 6 lines 3-4: "variant *C. difficile* PCR ribotype 027 contributed to the ever increasing number of cases" please substitute "in regions with epidemic outbreaks"*

The sentence was changed.

5. *page 6 line 14: "we here document the importance of PCR ribotype 001"please substitute "when analyzing the more recent isolates"*

The sentence was modified accordingly.

6. *Please add a comment into the discussion concerning differenced betwee the historical 027 strains detected in 1990 and the new epidemic outbreak strains.*
We have no detailed knowledge about these differences. However, we highlight the possibility to use isolates from 1990 for comparative analysis with recent outbreak strains (page 5, lien 3 ff: Interestingly, we were able to demonstrate presence of *cdtB*-positive *C. difficile* ribotype 027 isolates already in a collection of 23 strains from 1990, demonstrating that ancestors of the current epidemic clone were present in virulent *C. difficile* populations

almost 20 years ago. Detailed molecular comparison of these early strains with current *C. difficile* 027 isolates could provide new insights into the evolution of this particular strain, which is a prerequisite for understanding the molecular basis for its epidemic potential.”

7. *A direct comparison of antibiotic susceptibility and binary toxin between the 1990 and 2008 strains with the same ribotypes may be of interest*

We agree with the referee. However, we think that due to the small numbers of certain ribotypes this analysis in our collection is not meaningful.

8. *Page 6 line 22: "The rare occurrence of cdtB in our and independent C. difficile collections [8,11] shows that the ability to produce binary toxin is of minor importance for increase of CDI rates," The study design is not appropriate to give a statement about the clinical importance of binary toxins. Only descriptive analysis is allowed.*

To cope with this point of criticism we have changed the sentence, removing the statement related to our findings.

Reviewer 2

The term fluorquinolon was corrected throughout the text.

Page 5, line 1.

The authors should consider inserting the 87% proportion as stated in the abstract, to describe the number of isolates susceptibles to both clones. Otherwise, the 87% proportion cannot be found in the main text of the manuscript.

We followed the suggestion. page 3, line 24 ff: “.....while 20 / 23 (87 %) were susceptible against moxifloxacin and erythromycin (Table 1).”

Page 5, line 18

For the same reason as above, 87% should be mentioned again in this sentence, so that it is clear which are the two proportions that are being compared. A suggestion:

"Overall, compared with C difficile strains from 1990 the proportion of erythromycin and moxifloxacin susceptible strains was significantly lower in C.difficile isolates from 2008 (20/23, 87% vs 27/73, 37%, $p < 0.001$).

We followed the suggestion.