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Summary of the results of Clostridium difficile investigations

Introduction

During the last ten years, the increased incidence and outbreaks of C. difficile infection (CDI) are getting more and more important. This trend has been partly explained with the emergence of PCR ribotype 027/NAP1/toxinotype III, increased consumption of broad-spectrum antibiotics among hospitalized patients and in the community, the ageing population, increasing number of immunocompromised patients and naturally, the development of laboratory diagnostic possibilities (Weiss et al., 2007). Numerous theories have been postulated to explain the increasing number of CDI outbreaks, including the increased antibiotic consumption mainly fluoroquinolones, macrolids, and β-lactams (Pepin et al., 2005; Loo et al., 2005). At the same time, Weiss et al. showed that CDI outbreaks in five Quebec institutions between 2001 and 2004 were mostly associated with poor infection control (Weiss et al., 2007). The increased antibiotic consumption may have impact on the emergence of multidrug-resistant C. difficile strains. These strains may carry various genes coding for resistance to different antibiotics.

In Hungary, we have followed the prevalence of CDI since 2001. In 2007, the first case of sporadic CDI caused by PCR ribotype 027 could be detected; after this time, no cases associated with the presence of this PCR ribotype could be identified. During that time, we showed that the prevalence of toxin-positive and binary toxin-producing strains has been increased (3.8% of toxin A and B-positive strain carried cdtB gene in 2002-2003, while 6.7% in 2006 and 2007) (Terhes et al., 2009). Antibiotic susceptibility testing of 80 C. difficile strains and further 20 C. difficile strains isolated earlier against 5 antibiotics was also performed and we selected 5 strains which showed high level resistance to rifampin, erythromycin, clindamycin and moxifloxacin. On the basis of these facts, in our research plans were to follow changes in antibiotic susceptibility of toxigenic C. difficile strains isolated from diarrhoeal stools, and to characterize the resistant isolates, to continue the
epidemiological surveys about the spread of various *C. difficile* strains, and to determine their prevalence.

**MATERIALS AND METHODS**

During the study periods (May, 2010 and April, 2013), 1054 of *C. difficile* strains were collected from various parts of Hungary represented West, East and South of Hungary and the capital area (Budapest). These strains were isolated from diarrhoeal stools as part of the routine microbiological investigations. Laboratories used selective media such as, cycloserine-cefoxitine fructose (Cycloserine Cefoxitin Fructose Agar, Oxoid, UK) and Brazier’s CCEY agars (Cefoxitin/Cycloserine Egg Yolk, Mast Group, UK); at the same time some laboratories used alcohol shock method for selective isolation of *C. difficile* spores. The isolates were identified on the basis of characteristic colony morphology, smell, appearance on a Gram film or phase contrast microscopic preparation, and fluorescence under UV light. Toxin production was checked directly from diarrhoeal stool using commercial toxin assays. If this test was negative, but *C. difficile* was grown from stool specimen, the toxin production of the isolated strain was repeatedly checked from broth culture on the basis of two-step algorithm. After isolation, the strains were sent to our laboratory in an Amies transport medium without charcoal (Transwab® Amies, Medical Wire and Equipment, UK) for further characterization, including *tcdA/B* using NK2, NK3, NK104 and NK105 primer pairs (Kato, H. *et al.*, 1998; Kato, N. *et al.*, 1991) and binary toxin gene PCRs (Stubbs, S. *et al.*., 2000) and in the case of binary toxin positivity, we set up PCR ribotyping (Stubbs, S. *et al.*., 1999) to recognize the presence of PCR ribotype 027. If past medical history about the patient was available *tcdC* PCR was set up using the primer published by Spigaglia and Mastrantonio (2002). 200 of toxin-positive *C. difficile* strains were selected from these isolates for antibiotic susceptibility testing. Because of the high number of PCR ribotype 027 strains among the examined strains, we tried to avoid over-representation of this PCR ribotype in the case of antibiotic susceptibility testing, thus only in two cases, PCR ribotype 027 strains were selected for this investigation. Antibiotic susceptibility testing was performed using MIC Test Strip (Liofilchem, Italy). Colonies of *C. difficile* grown on anaerobic blood agar were suspended into brain heart infusion broth to achieve the turbidity equivalent to 1 McFarland. The suspension was swabbed on prereduced Brucella agar supplemented with blood (5 %), haemin (5 µg/ml), and vitamin K1 (1 µg/ml). The plates were incubated in an anaerobic
cabinet (85 % N₂, 10 % CO₂, 5 % H₂, Concept 400, anaerobic workstation, Ruskinn Technology Limited, Bridgend, UK) for 24-48 h at 36 °C. The following antibiotics were tested: erythromycin, clindamycin, moxifloxacin, metronidazole, and rifampin. Breakpoints for clindamycin and metronidazole were applied on the basis of CLSI recommendations for anaerobic bacteria (CLSI, 2011). In the case of erythromycin and rifampin, CLSI interpretative criteria for Staphylococcus aureus were used (CLSI, 2011), while breakpoints for moxifloxacin were applied on the basis of Ambler et al. (2008). Quality control strain (B. fragilis ATCC 25285) was used to validate the result of susceptibility testing.

Simultaneously, DNA extraction was performed from the tested strains for further characterization using solution of Chelex-100 (Bio-Rad, Hemel Hempstead, UK). In the case of erythromycin resistance, the presence of ermB gene was tested according to Eitel et al. (2013). The sequences of the primers used for PCR amplification for the detection of ermB gene were as followed: ermB_F– 5’-GCG GAA TGC TTT CAT CCT AA-3’ (primer Tm: 60.2 °C); and ermB-R 5’-GCG TGT TTC ATT GCT TGA TG-3’ (primer Tm: 60.3 °C). The reaction mixture (20 μl) contained 2 μl of template DNA, 10 μl of Maxima Sybr Green qPCR Master Mix (2x) (Thermo Scientific), 0.3 μl of ermB_1_F and 0.3 μl of ermB_2_F, 0.4 μl of 25 mM MgCl₂, and 7 μl of nuclease-free water. The reaction mixtures were incubated for 35 cycles in Step OneTM Real-time PCR System (Life Technologies). Gene amplification consisted of denaturation at 95 °C for 15 s, annealing at 60 °C for 30 s, and extension at 72°C for 30 s, respectively. Starting denaturation was performed at 95 °C for 10 min, while melting curve was produced at 95 °C for 15 s, followed by 60 °C for 1 min, and 95 °C for 15 s. In the case of each run, PCR was also set up with negative control (nuclease-free water) and positive control (C. difficile strain 630 genomic DNA). During the analysis, we checked the Tm of the amplified PCR product.

RESULTS AND DISCUSSION

During the study period, 1054 of C. difficile strains were arrived in our laboratory for further characterizations. 194 of these samples were excluded from this study, because of highly contaminated culture or C. difficile was not grown. 860 strains were characterized and we checked the toxigenic status of these strains. 54 of the tested strains proved to be negative for the presence of toxins A and B and these strains were also negative for the presence of
binary toxin genes. In the case of 303 *C. difficile* strains, *tcdA* and/or *B* genes were detected, while these strains did not carry binary toxin genes. 2 strains were toxin A-negative and toxin B-positive, however during the last ten years this type of toxin-variant strains could not be found. During the *tcdC* analysis, we could not find connection between the deletions and the severities of CDI. 501 strains harboured *tcdA*, *tcdB* and binary toxin genes, and the majority of these strains (490 isolates of 501 tested *C. difficile* strains) proved to be PCR ribotype 027. Thus 56.9 % of the tested *C. difficile* strains belonged to PCR ribotype 027! When we first recognized the outbreak situation of PCR ribotype 027 in June, 2010, and we sent back the results of further characterization to the laboratories participating in this study, the number of *C. difficile* strains sent to our laboratory increased within a short period of time. We tried to advertise and show the outbreak situation in Hungarian symposia and because of the high number of recognised CDI, an official Hungarian guideline for the diagnosis and prevention of CDI was edited. Because of the high workload, introduction of real-time PCR method for the detection of major toxin genes and binary toxin genes was necessary. We successfully used the primers described by Kato *et al.* (Kato, H. *et al.*, 1998; Kato, N. *et al.*, 1991) and Stubbs *et al.* (Stubbs, S. *et al.*, 2000) in real-time PCR assay with modifications in the master mix compositions and on the basis of melting analysis, we can recognise the specific PCR products. Real-time PCR was set up directly from stool after DNA isolation using QIAamp DNA mini kit (Qiagen) and from isolated strain after DNA isolation using Chelex-100 (BioRad). We also compared the results of home-made real-time PCR with the results produced by EasyScreen *C. difficile* Reflex kit and EasyScreen *C. difficile* detection Kit (Human Genetic Signatures). In these cases, the home-made real-time PCR gave more reliable results than the commercially available assays. For antibiotic susceptibility testing, 200 toxins A and B positive strains were selected. Among the tested isolates, 6 strains harboured the binary toxin genes, 2 of them were PCR ribotype 027. MICs of 200 toxigenic *C. difficile* were determined using MIC Test Strip. 5 antibiotics were tested; these are erythromycin, clindamycin, moxifloxacin, metronidazole, and rifampicin. We compared these results with earlier findings in Hungary. All of the tested 200 strains were susceptible to metronidazole in this study period. The same results were found in the case of Hungarian *C. difficile* strains isolated in 2002-2003 (20 tested strains) and 2006-2007 (80 tested strains). MIC ranges were 0.032-2 μg/ml in the tested strains. In 2006-2007, MIC$_{50}$ was 0.25 μg/ml, while in the present study, this slightly increased (MIC$_{50}$ 0.5 μg/ml). Resistance rates to
Erythromycin, clindamycin, moxifloxacin, and rifampicin were 31%, 29.5%, 21.5%, and 11.5%, respectively. While between 2006 and 2007, 25% of the tested strains proved to be resistant to erythromycin, and moxifloxacin, and 27.5% of these strains were resistant to clindamycin. In the case of these antibiotics, MIC<sub>50</sub> and MIC<sub>90</sub> were not changed, and only small increase in the resistance rates to erythromycin and clindamycin could be observed. At the same time, 25.5% and 16% of the tested strains showed intermediate resistance to erythromycin and clindamycin, respectively. The MICs of rifampicin were either high or low, and this tendency became more obvious from 2008 to 2012. Furthermore, the majority of strains (88%) showed susceptibility to rifampicin (MIC ≤0.002 μg/l) and 11.5% of strains were proven to be resistant. 20 strains among rifampicin resistant isolates were also resistant to erythromycin, clindamycin, and moxifloxacin, while only 1 strain showed rifampicin resistance. The number of strains tested and the prevalence of genes conferring antimicrobial resistance (ermB) in resistant isolates were as follows. We selected erythromycin resistant strains and in 2008, out of 16 selected erythromycin resistant strains, which exhibited resistance against erythromycin, only 11 carried the ermB gene. In 2009, 23 erythromycin resistant strains were examined and 21 strains were positive for ermB, while 2 strains which had high level erythromycin resistance phenotype, did not carry this resistance gene. In 2010, 12 erythromycin resistant strains were tested, all of them were positive for ermB. Out of 20 strains which exhibited combined resistance against MLSB, MFX, and RIF, we could test 18 strains about the presence of ermB gene; the prevalence of ermB gene was 16 (88.9%). The prevalence of ermB gene in strains resistant to MLSB only (29 strains tested out of 35 strains) was 19 (66%).

In conclusion, during the last ten years, we experienced that the epidemiology of CDI has changed from time to time. The prevalence of toxin variant strains including binary toxin-producing and toxin A-negative, toxin B-positive strains has been increased. As in other European countries, we faced with outbreak situation of PCR ribotype 027 and unfortunately, up to this point we can not solve this problem, however official Hungarian guideline is available, CDI is notifiable disease, and strong infection control measures were introduced. On the basis of the results of antibiotic susceptibility tests, the prevalence of rifampicin resistant isolates were increased, this may be explained by the fact that rifaximin is used more frequently for the therapy of CDI.
References


Poster presentations related to this grant


**Terhes Gabriella**, Sóki József, Latkóczi Krisztina, Szikra Lenke, Konkoly-Thege Marianne, Princz Gyula, Nagy Tünde, Osztie Hilda, Urbán Edit

Emergence and distribution of *Clostridium difficile* PCR ribotype 027 in Hungarian hospitals.

**Clinical Microbiology and Infection** 17:(S4) pp. 228-229. (2011)

21st ECCMID/27th ICC Milan, Italy 7-10 May, 2011

**Terhes Gabriella**, Sóki József, Latkóczi Krisztina, Szikra Lenke, Konkoly-Thege Marianne, Princz Gyula, Nagy Tünde, Osztie Hilda, Urbán Edit

Antibiotic susceptibility pattern of nosocomial and community-acquired *Clostridium difficile* in Hungary.

**Clinical Microbiology and Infection** 17:(S4) p. 291. (2011)

21st ECCMID/27th ICC Milan, Italy 7-10 May, 2011


Changes in the epidemiology and antibiotic susceptibility pattern of nosocomial and community-acquired *Clostridium difficile* in Hungary

4th International *Clostridium difficile* Symposium, Bled, Slovenia, 20-22 September 2012.

**Gabriella Terhes**, Elizabeth Nagy, Edit Urbán

Prevalence of gastrointestinal disease and clinical features of *Clostridium difficile*-associated infections: results of a retrospective study: 2000-2010 in a University Hospital in Hungary

4th International *Clostridium difficile* Symposium, Bled, Slovenia, 20-22 September 2012.


Magyarországi *Clostridium difficile* törzsek antibiotikum érzékenységének változása (Changes in the antibiotic susceptibility pattern of Hungarian *Clostridium difficile* strains) p. 58.


**Lectures related to this grant**

**September 30-October 2, 2010**

Hazai *Clostridium difficile* törzsek toxintemelési sajátságai és antibiotikum érzékenysége (Toxin production and antibiotic susceptibility of Hungarian *Clostridium difficile* strains)

MIFKMT 38. Kongresszusa, Debrecen (38th Congress of Hungarian Society for Infectology and Clinical Microbiology, Debrecen, Hungary)

**September 22-24, 2011**

*Clostridium difficile* törzsek toxintemelésének és antibiotikum-érzékenységének vizsgálata (Investigation of toxin production and antibiotic susceptibility of *Clostridium difficile* strains)

MIFKMT 39. Kongresszusa, Pécs (39th Congress of Hungarian Society for Infectology and Clinical Microbiology, Pécs, Hungary)

**June 3-8, 2012**

What is new in *C. difficile* infections; epidemiology, diagnostics and therapy?

“Anaerobes in health and disease; how to isolate, identify and look for resistance in a cost effective way” ESCMID Postgraduate course/Technical workshop, Szeged, Hungary