IMPLEMENTATION OF MOLECULAR METHODS FOR IDENTIFICATION, DETECTION OF TOXIN ENCODING GENES AND TYPING IN *CLOSTRIDIUM DIFFICILE*

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INTRODUCTION

Clostridium difficile **(*CD*) is associated with asymptomatic colonization, mild to severe diarrhea and life-threatening gastrointestinal diseases. It is considered a causative agent in 25% of the total cases of antibiotic-associated diarrhea (CDAD) and most cases of pseudomembranous colitis. Asymptomatic carriers are an important hidden reservoir of *CD.***

The main predisposing factors for *C. difficile* infection (*CDI*) include high age, hospitalization, immune-compromising conditions and exposure of antimicrobial agents.

The enteropathogenicity of *CD* is mediated by two toxins: enterotoxin A (TcdA) and cytotoxin B (TcdB). Some strains of clinical significance also produce a third chromosomally encoded binary toxin (CDT) (9).

The enzyme glutamate dehydrogenase (GDH) is expressed at high levels by all *CD* strains and is referred to as common antigen (10,13).

Genotyping methods such as PCR-ribotyping and Multilocus Variable-Number Tandem Repeat Analysis 7 (MLVA7) are used for characterization of *CD* strains (7, 8).

MATERIALS AND METHODS

We have analyzed 110 stool samples from patients with mild to severe enterocolitis and history of previous antibiotic therapy. Isolation and identification of *CD* was performed by standard microbiological techniques and latex-agglutination test (Culturetted, BD, USA).

Toxin A and B production was analyzed by Immuno Card Toxins A&B-EIA (Meridian, Biosciense,USA). Bacterial DNA was isolated by QIAamp DNA Mini Kit (Qiagen). Detection of toxin genes *tcdA*, *tcdB*, *cdtA* and *cdtB* and *gluD* was performed by EvaGreen Real-time PCR and confirmed by melting curve analysis.

PCR-ribotyping and MLVA7 were applied for genotyping of the strains. Ribotype patterns were compared to reference the 25 ECDC *CD* collection.

Table 1*: *Primers and parameters used in PCR-methods

Target gene	*Primers/ sequences*	*Primer concentration [µM]*	*Temp/ time annealing [^{0}C]*	*Temp/ time Extension [^{0}C]*	*Size (bp)*
gluD	**GluD-s** 5'-GTCTTGGATGGTTGATGAGTAC-3 **GluD-as** 5'-TTCCTAATTTAGCAGCAGCTTC-3 ' (10)	0,4	58^O C - 25 s	72^0 C - 30 s	158
tcdA	**Tox-A-s** 5'-TGTTGGAATAGGTGCTGAAG-3' **Tox-A-as** 5'- AGATGGAGATGAGAAAAAGTGA- 3' (in house primers, ECDC)	0,4	53^0 C - 35 s	70^0 C - 30 s	331
tcdA	**NKV 011** 5'-TTTTGATCCTATAGAATCTAACTTAGTAAC- 3' **NK 9** 5'- CCACCAGCTGCAGCCATA- 3' (6)	0,4	60^0 C - 30 s	65^0 C - 3 min	2535 714
tcdB	**NK104** 5'- GTGTAGCAATGAAAGTCCAAGTTTACGC- 3' **NK105** 5'- ACTTAGCTCTTTGATTGCTGCACCT-3' (5)	0,4	58^0 C - 30 s	72^0 C - 30 s	204
cdtA *cdtB*	**cdtA- fw** 5'-TGAACCTGGAAAAGGTGATG-3' **cdtA- rev** 5'- GGATTATTTACTGGACCATTTG-3' **cdtB-fw** 5'- CTTATTGCAAGTAAATACTGAG- 3' **cdtB-rev** 5'- ACCGGATCTCTTGCTTCAGTC- 3' (3)	0,15 0,5	54^0 C – 30 s	72^0 C - 30 s	376 510

Real-time PCR was performed on IQ5 system (Bio-Rad). Fluorescent readings were performed during elongation step. Melting curve analysis was performed in range 72-95^{0}C in 0,5^{0}C/s steps.

Discrimination of A+/B+ and A-/B+ *CD* isolates was performed by PCR with primers NKV011/NK9 targeting a specific deletion in *tcdA* gene (714bp). Presence of this deletion leads to production of inactive toxin A (6).

***CD* carries multiple copies of the rRNA genes, which vary not only in number between strains but also in size between different copies on the same genome (2, 8). PCR-ribotyping is based on the amplification of multiple alleles of the rRNA operon and was applied as a typing method coupled with fragment analysis on capillary gel electrophoresis. Specific primers complementary to 3'-end of the 16S rRNA gene and to 5'-end of the 23S rRNA gene was used to amplify the variable-length inter-spacer region:**

16S 5'- GTGCGGCTGGATCACCTCCT- 3'

23S 5'- CCCTG CACCCTTAATAACTTGACC- 3' (2)

PCR were performed according to Bidett at al. (1999).

The fragments were separated either by QIAxcel capillary electrophoresis system (Qiagen) (NCIPD, Bulgaria) or by regular agarose electrophoresis (CDRL, Leiden). Electrophoretic ribotype patterns were compared to reference the 25 ECDC *CD* collection.

For genotyping of *CD* strains we used Multiple-locus VNTR analysis (MLVA), which is based on a set of seven polymorphic tandem repeat loci. All loci were amplified in 3 duplex ($A6_{Cd}$ - $H9_{Cd}$; $B7_{Cd}$ - $F3_{Cd}$; $C6_{Cd}$ - $E7_{Cd}$) and 1 single PCR ($G8_{Cd}$) (table 2; 11, 12).

Table 2.* *Primers and conditions used in MLVA7 analysis. Fragment analysis was performed on QiAxcel capillary system and sizes converted to repeat copy numbers was exported to Bionumerics v4.5 as character data set. Clustering and dendrogram construction was performed by using the categorical coefficient and UPGMA respectively.

Marker	*Repeat motif*	Primers	*Primer concentration [µM]*	*Temp/ time annealing [^{0}C]*	*Temp/ time Extension [^{0}C]*
$A6_{Cd}$	AAGAGG	5'-TTAATTGAGGGAGAATCTTAAA- 3' 5'- AAATACTTTTCCCACTTTCATAA- 3'	1,5	51^{O}C - 30 s	71^0 C - 75 s
$H9_{Cd}$	TCTTCTTCC	5'-GTTTTGAGGAAACAAACCTATC -3' 5'- GATGAGGAAATAGAAGAGTTCAA-'3' (11)	0,25		
$B7_{Cd}$	ATCTTCT	5'-CTTAATACTAAACTAACTCTAACCAGTAA -3' 5'-TTATATTTTATGGGCATGTTAAA -3'	1,5	50^0 C - 35 s	71^0 C - 45 s
$F3_{Cd}$	TTA	5'- TTTTTGAAACTGAACCAACATA- 3' 5'-ACAAAAGACTGTGCAAATATACTAA -3' (11)	0,7		
$C6_{Cd}$	TATTGC	5'- GTTTAGAATCTACAGCATTATTTGA- 3' 5'- ATTGGAATTGAATGTAACAAAA -3'	1,5	50^0 C - 35 s	71^0 C - 75 min
$E7_{Cd}$	ATAGATT	5'- TGGAGCTATGGAAATTGATAA -3' 5'- CAAATACATCTTGCATTAATTCTT -3' (11)	0,35		
$G8_{Cd}$	TAAAAGAG	5'- TGTATGAAGCAAGCTTTTTATT- 3' 5'-AATCCAGCAATCTAATAATCCA -3' (11)	1	50^0 C - 35 s	71^0 C - 90 s

RESULTS

Toxin production of toxin A/B by EIA was registered in 27,3% (30/110) of the stool samples whereas culture isolation rate was higher 33,6% (37/110). *GluD* gene however was confirmed in only 27 of the 37 isolates indicating a significant false positive results in EIA and culture (*fig. 4*).

***TcdA* was determined in 88,9% (24/27) with primers Tox-A-s/Tox-A-as, while with NKV011/NK9 29,6% (8/27) of the strains harbored the *tcdA* deletion (714bp) (*fig. 1* and *fig. 2*). TcdB gene was observed in 96,3% (26/27) (*fig. 3*). We have developed multiplex PCR method for detection of *tcdB* and *gluD* genes. The sensitivity of the method was 24 CFU for *gluD* and 2,4 CFU for *tcdB* respectively (*fig. 5*). Three toxin variants were distinguished among our clinical isolates: 62,96% (17/27) toxin A+B+CDT- ; 29,62% (8/27) A–B+CDT- ; 7,4% (2/27) A+B-CDT+ . The binary-toxin genes *cdtA*/*cdtB* were detected in two of the A+B+ strains (*fig. 6*).**

Eight ribotypes were confirmed and the most prevalent one was 017- 29,6% (8/27) followed by 014/020– 18,5% (5/27), 001, 002; 012 by 7,4% and 046, 070, 078 represented by 3,7% each. Eighteen percent of *CD* (5/27) corresponded to non-reference PCR- ribotypes (*fig.7* and *table 3*).

A total of 19 MLVA7 genotypes were detected in our strains, distributed as follows: four for ribotype 017; two for 001, 002, 012, 014/020 respectively; one for ribotype 046, 070 and 078 respectively. Being more discriminative the MLVA typing could supplement the ribotyping assisting in outbreak investigations (fig. 8).

A cluster of cases infected with ribotype 017 and MLVA7 type 018 were registered in a hospital in Sofia. This genotype was associated with significant complications and high mortality rates (fig. 8).

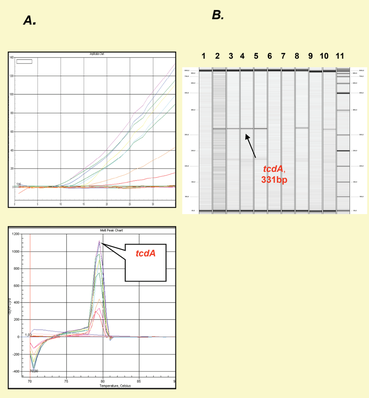

Fig. 1: **Detection of *tcdA gene with* Tox-A-s/Tox-A-as *primers in CD clinical strains.* *A.*** Real time PCR*; **B.*** Capillary gel electrophoresis: **1**- 239; **2**- 181; **3**- 1795-9; **4**- 1797-15; **5**- 217; **6**- 257; **7**- 367; **8**- 253; **9**- negative control, ddH20; **10**- ribotype 002 (ECDC) possitive for *toxA* gene; **11**- DNA marker, 50-1000 bp

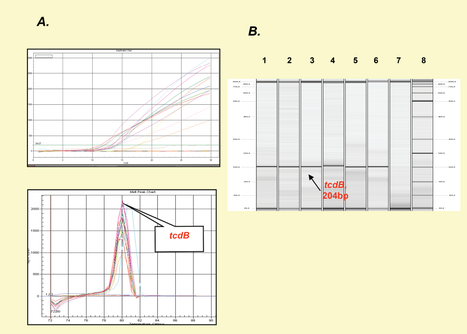

Fig. 3: ***Detection of tcdB gene with NK104/NK105 primers in CD clinical strains.***
A. Real time PCR*; **B.*** Capillary gel electrophoresis: **1**- 36; **2**- 250; **3**- 253; **4**- 256; **5**- 262; **6**- ribotype 002 (ECDC);**7**- negative control, dd H2O; **8**- DNA marker, 50-1000 bp

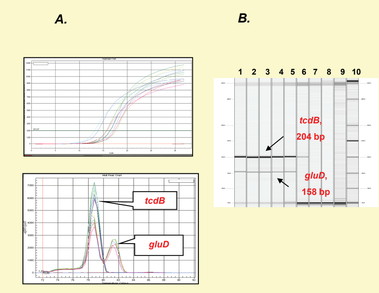

Fig. 5: **Multiplex PCR for *detection of tcdB and gluD genes in CD***
A. Real time PCR*; **B.*** Determining susceptibility of the method with **RS 027:**
1- 24.10 ^{4} CFU/ µl; **2**- 24.10 ^{3} CFU/ µl; **3**- 24. 10 ^{2} CFU/ µl; **4**- 24.10 ^{1} CFU/ µl; **5**- 24 CFU/ µl; **6**- 2,4 CFU/ µl; **7**- 2,4. 10 $^{-1}$ CFU/ µl; **8**- 2,4. 10 $^{-2}$ CFU/ µl; **9**- negative control dd H2O; **10**- DNA marker, 50-500 bp

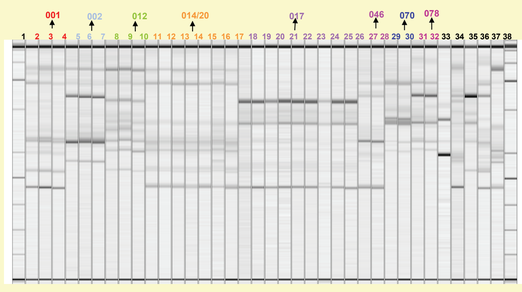

Fig. 7: **PCR- ribotyping of *CD* clinical strains: 1**-DNA marker 50-750 bp; **2**- RS 001 (ECDC collection); **3**- 302; **4**-322; **5**- RS 002; **6**- 262; **7**- 240; **8**- RS 012; **9**- 225; **10**- 333; **11**- RS 014; **12**-RS 020; **13**- 253; **14**- 256; **15**- 257/1; **16**- 301; **17**-355; **18**- RS 017; **19**- 36/1; **20**-181/1; **21**-217; **22**-237; **23**-238; **24**- 239; **25**-250; **26**- 367; **27**- RS 046; **28**- 1797-15; **29**- RS 070; **30**- 386; **31**- RS 078; **32**- 1795-9; **33**- 223; **34**- 228; **35**- 304; **36**-325; **37**- 59/07; **38**- DNA marker 50-750 bp

Table 3. **Ribotype distribution in Bulgarian *CD* isolates**

Ribotype (n = 27 isolates)	*Percent out of all determined ribotypes (%)*
001 (2)	7,4
002 (2)	7,4
012 (2)	7,4
014/020 (5)	18,5
017 (8)	29,6
046 (1)	3,7
070 (1)	3,7
078 (1)	3,7
UNK. (5)	18,5

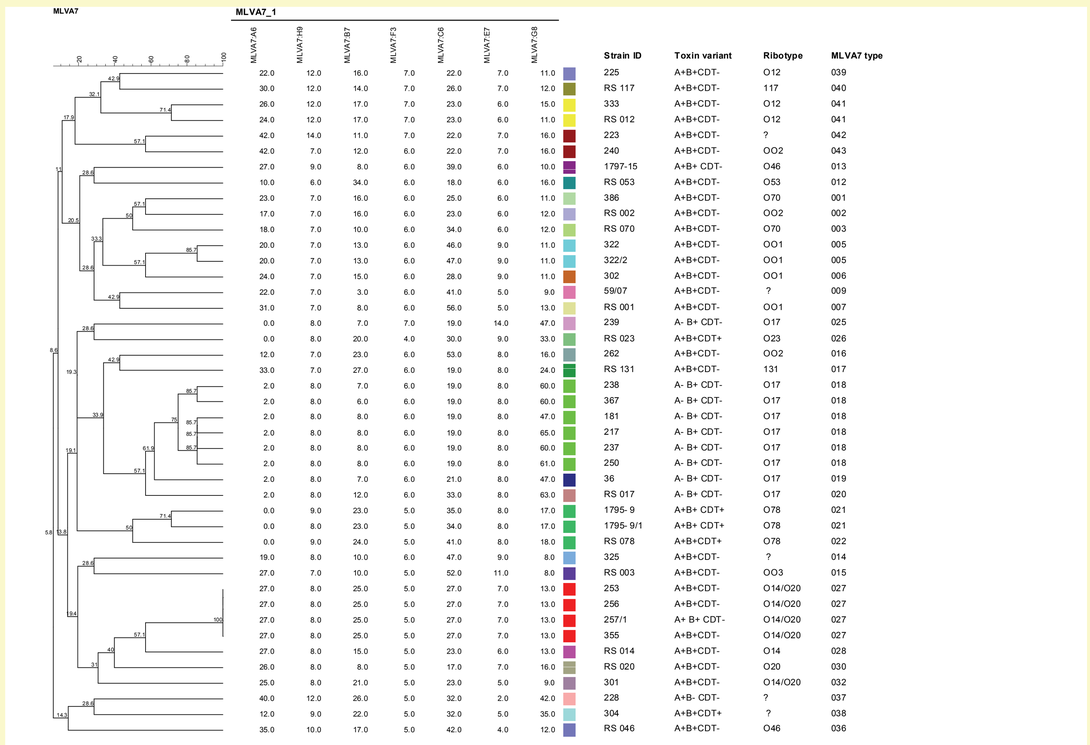

***Fig. 8*: MLVA7 typing method**

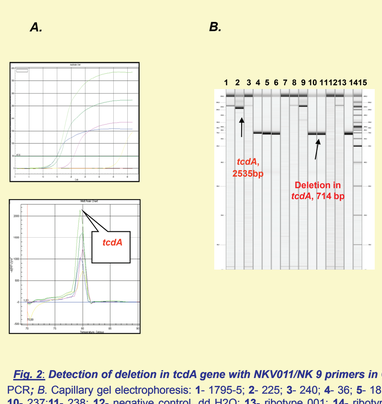

Fig. 2: ***Detection of deletion in tcdA gene with NKV011/NK 9 primers in CD clinical strains.*** ***A.*** Real time PCR*; **B.*** Capillary gel electrophoresis: **1**- 1795-5; **2**- 225; **3**- 240; **4**- 36; **5**- 181; **6**- 217; **7**- 223; **8**- 253; **9**- 256; **10**- 237;**11**- 238; **12**- negative control, dd H2O; **13**- ribotype 001; **14**- ribotype 017; **15**- DNA marker, 100bp-3000bp

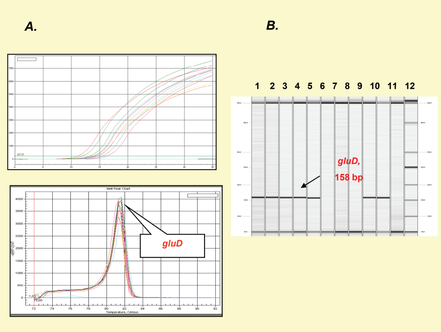

Fig. 4: ***Detection of gluD gene with primers GluD-s/ GluD-as in CD clinical strains.*** **A.** Real time PCR*; **B.*** Capillary gel electrophoresis: **1**- 181; **2**- 1795-9; **3**- 1797-15; **4**- 253; **5**- 257; **6**- 265; **7**- 266; **8**- 267; **9**- 304; **10**- RS 053; **11**- negative control dd H2O; **12**- DNA marker, 50-1000 bp

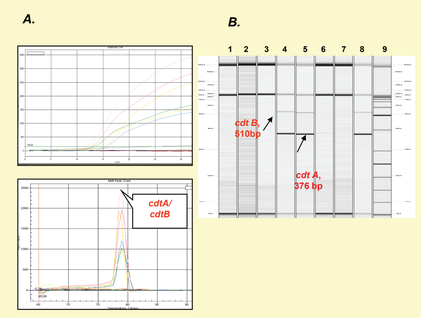

Fig. 6: ***Detection of cdtA and cdtB genes with cdtA-fw/cdtA-rev and cdtB-fw/ctdB-rev primers in CD clinical strains.*** ***A.*** Real time PCR*; **B.*** Capillary gel electrophoresis:
1- ribotype 003 (ECDC); **2**- ribotype 015; **3**- ribotype 056; **4**- ribotype 027; **5**- ribotype 023; **6**- 36; **7**- 181; **8**- 1795-9; **9**- DNA marker, 100- 3000 bp

CONCLUSIONS

The significant number of diagnosed *CD* cases with outbreak ribotypes might represent a problem in our country in the future. The results of the present study would support and improve the diagnostic and therapeutic preparedness of the Bulgarian hospitals when dealing with CDI.

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