Advances in molecular surveillance of *Clostridium difficile* in Bulgaria

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The increasing incidence of *Clostridium difficile* infection (CDI) in Bulgaria has indicated the need to implement better surveillance approaches. The aim of the present work was to improve the current surveillance of CDI in Bulgaria by introducing innovative methods for identification and typing. One hundred and twenty stool samples obtained from 108 patients were studied over 4 years from which 32 *C. difficile* isolates were obtained. An innovative duplex EvaGreen real-time PCR assay based on simultaneous detection of the *gluD* and *tcdB* genes was developed for rapid *C. difficile* identification. Four toxigenic profiles were distinguished by PCR: A$^+$ B$^+$ CDT$^+$ (53.1%, 17/32), A$^-$ B$^+$ CDT$^-$ (28.1%, 9/32), A$^+$ B$^+$ CDT$^+$ (9.4%, 3/32) and A$^-$ B$^-$ CDT$^-$ (9.4%, 3/32). PCR ribotyping and multilocus variable number of tandem repeat analysis (MLVA7) were used for molecular characterization of the isolates. In total, nine distinct ribotypes were confirmed and the most prevalent for Bulgarian hospitals was 017 followed by 014/020, together accounting for 44% of all isolates. Eighteen per cent of the isolates (6/32) did not match any of the 25 reference ribotypes available in this study. Twenty-four MLVA7 genotypes were detected among the clinical *C. difficile* isolates, distributed as follows: five for 017 ribotype, two for 014/020, 001, 002, 012 and 046 each, and one each for ribotypes 023, 070 and 078. The correlation between the typing methods was significant and allowed the identification of several clonal complexes. These results suggest that most *C. difficile* cases in the eight Bulgarian hospitals studied were associated with isolates belonging to the outbreak ribotypes 017 and 014/20, which are widely distributed in Europe. The real-time PCR protocol for simultaneous detection of *gluD* and *tcdB* proved to be very effective and improved *C. difficile* identification and confirmation of clinical *C. difficile* isolates.

INTRODUCTION

*Clostridium difficile* is a well-known causative agent of infections in humans and animals worldwide. The spectrum of *Clostridium difficile* infection (CDI) varies from mild diarrhoea to severe colitis including pseudomembranous colitis, toxic megacolon, perforation, sepsis and death (Bartlett & Perl 2005).

*C. difficile* is an important problem in healthcare facilities, because it is easily transmitted via the faecal–oral route from the hands of healthcare workers to patients and to the environment (McFarland et al. 1989). The main predisposing factors for contracting *C. difficile* include advanced age, hospitalization, immune-compromising conditions and exposure to antimicrobial agents (McFarland 1998).

Since the Pan-European surveillance study conducted in 2008, the incidence of CDI in Bulgarian hospitals has increased from 3 to 7.94 per 10,000 patient admissions (Bauer et al. 2011), although it is believed that these numbers barely reflect the real picture. Currently, national reporting of CDI is not mandatory in Bulgaria, and most hospitals have only recently adopted diagnostic services for CDI.

The aim of the present work was to improve the current surveillance of CDI in Bulgaria by introducing innovative methods for identification and typing.

METHODS

Uniformed stool samples (n=120), obtained from 108 patients admitted to eight hospitals in Sofia and one in Plovdiv, Bulgaria, were analysed over the period November 2008 to November 2012. The accepted criteria for CDI were fulfilled for all patients: age >2 years, mild to severe diarrhoea or antibiotic-associated diarrhoea and onset of diarrhoea after the third day of hospital admissions. The
ethics committee of each hospital approved the study and written informed consent for participation was obtained from all patients prior to sending samples to the National Centre of Infectious and Parasitic Diseases, Sofia, Bulgaria.

Stool samples were tested for toxins A and B directly with an Immuno Card Toxins A&B kit (Meridian Bioscience) as a first step in the diagnostic algorithm, and stool culture was used as a confirmatory method. Standard microbiological techniques were used to isolate Campylobacter and Enterobacteriaceae. C. difficile direct inoculation of the stool sample on cycloserine-cefoxitin-fructose selective agar (CCFA) (HiMedia) and culture on dehydrogenase common antigen and other undisclosed proteins agar after an alcohol shock procedure.

DNA was isolated from several suspicious colonies of each specimen using a QIAamp DNA Mini kit (Qiagen) according to the recommended protocol for tissues.

A novel duplex qualitative EvaGreen-based real-time PCR was developed and optimized for simultaneous detection of the *gluD* and *tdcB* genes using previously published primers (Table 1). Briefly, the PCR mix (25 μl) consisted of: 1 × PCR buffer [50 mM Tris/HCl (pH 9.2), 2.5 mM MgCl₂, 0.1% Tween 20], 0.2 mM dNTP, 0.5 U Hot-Start CesiumTaq (Vivantis), 2 μl DNA (~50 ng) and optimized concentrations of primers (Table 1). Real-time PCR was performed on an iQ5 system (Bio-Rad) with initial denaturation at 95 °C for 4 min, followed by 40 cycles of 94 °C for 30 s, 58 °C for 30 s and 72 °C for 30 s for fluorescence acquisition during the extension step, with subsequent melting-curve analysis over 72–95 °C in 0.4 °C steps. Although primer specificity has been tested previously by others, we have tested the method on 25 reference ribotypes available in this study as well as on 24 non-related *C. difficile* ribotypes.

### Table 1. Primers and parameters used in the PCR methods

<table>
<thead>
<tr>
<th>Purpose/target gene</th>
<th>Primer sequence (5′→3′)</th>
<th>Primer conc. (μM)</th>
<th>Annealing °C</th>
<th>Extension °C</th>
<th>Size (bp)</th>
<th>Reference</th>
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<td>Identification</td>
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<tr>
<td><em>gluD</em></td>
<td>GTCTTGATGTTGATGAGTAC</td>
<td>0.4</td>
<td>58 °C for 30 s</td>
<td>72 °C for 30 s</td>
<td>158</td>
<td>Paltansing et al. (2007)</td>
</tr>
<tr>
<td>Detection of toxin genes</td>
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<tr>
<td><em>tdcA</em></td>
<td>TGTGGAATGTTGCTGGAAGAG</td>
<td>0.4</td>
<td>53 °C for 35 s</td>
<td>70 °C for 30 s</td>
<td>331</td>
<td>ECDC</td>
</tr>
<tr>
<td></td>
<td>AGATGGGAGATGAGAAAAAGTGA</td>
<td></td>
<td></td>
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<tr>
<td><em>tdcB</em></td>
<td>TGTGAGAATGAAAGTCAATAGCT</td>
<td>0.6</td>
<td>60 °C for 30 s</td>
<td>65 °C for 3 min</td>
<td>2535</td>
<td>Kato et al. (1999)</td>
</tr>
<tr>
<td></td>
<td>AGATGGGAGATGAGAAAAAGTGA</td>
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<tr>
<td>Deletion</td>
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<td><em>tdcA</em></td>
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<td><em>tdcB</em></td>
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<td>Kato et al. (1999)</td>
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<td></td>
<td>AGATGGGAGATGAGAAAAAGTGA</td>
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<td>Typing</td>
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<tr>
<td>16S–23S</td>
<td>GTCCGGCGCTGAGTACCTCCTCT</td>
<td>0.35</td>
<td>60 °C for 35 s</td>
<td>72 °C for 90 s</td>
<td>Varies</td>
<td>Bidet et al. (2000)</td>
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<td>A6Cd</td>
<td>TTAATTTAGGAGAGATCTCTAAA</td>
<td>1.5</td>
<td>51 °C for 30 s</td>
<td>71 °C for 75 s</td>
<td>Varies</td>
<td>Van Den Berg &amp; Schaad et al. (2006)</td>
</tr>
<tr>
<td>H9Cd</td>
<td>GTTGGAGAAAAACACTATC</td>
<td>0.25</td>
<td>51 °C for 30 s</td>
<td>71 °C for 75 s</td>
<td>Varies</td>
<td>Van Den Berg &amp; Schaad et al. (2006)</td>
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<td>B7Cd</td>
<td>CTATATTTACACTACTTAACGTAATTTATTTTATGGCATGTTAAA</td>
<td>1.5</td>
<td>50 °C for 35 s</td>
<td>72 °C for 45 s</td>
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<td>F3Cd</td>
<td>TTTTTGAAAGACACAAAATTTTCAAAGGAATGGCAATATACTAA</td>
<td>0.7</td>
<td>50 °C for 35 s</td>
<td>72 °C for 45 s</td>
<td>Varies</td>
<td>Van Den Berg &amp; Schaad et al. (2006)</td>
</tr>
<tr>
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<td>1.5</td>
<td>50 °C for 35 s</td>
<td>72 °C for 45 s</td>
<td>Varies</td>
<td>Van Den Berg &amp; Schaad et al. (2006)</td>
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<tr>
<td>E7Cd</td>
<td>TGGGCTATGGAAATTGCTAA</td>
<td>0.35</td>
<td>50 °C for 35 s</td>
<td>72 °C for 45 s</td>
<td>Varies</td>
<td>Van Den Berg &amp; Schaad et al. (2006)</td>
</tr>
<tr>
<td>G8Cd</td>
<td>1</td>
<td>50 °C for 35 s</td>
<td>72 °C for 90 s</td>
<td>72 °C for 45 s</td>
<td>Varies</td>
<td>Van Den Berg &amp; Schaad et al. (2006)</td>
</tr>
</tbody>
</table>

Note: All primers were designed to amplify fragments of the gene of interest, and the PCR conditions were optimized to ensure specific amplification and melting-curve analysis. The PCR products were visualized on an agarose gel and analyzed by melting-curve analysis. The results were consistent with previously published methods.
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(a) PCR baseline subtracted curve fit (RFU)

(b) -d(RFU)/dT

(c) Gel electrophoresis

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PCR kit (Qiagen) was applied to improve the amplification of target fragments. A non-sequencer-based QIAxcel capillary gel electrophoresis system (Q-CGE; Qiagen) was used for analysis using the MS500 method on a high-resolution cartridge. The obtained profiles were compared with the profiles of 25 reference ribotypes provided by ECDC. A modified multilocus variable number of tandem repeat analysis (MLVA7) scheme (Van Den Berg et al. 2007) (Table 1) was used for subtyping using unlabelled primers in single PCRs and products resolved by Q-CGE. Fragment analysis was performed using Biocalculator v3.2 software (Qiagen) and sizes were converted to repeat copy numbers and exported to BioNumerics v4.5 as character dataset. The cluster analysis was performed using the categorical (or Hamming’s) distance and UPGMA (unweighted pair group method with arithmetic mean) algorithms. A minimum spanning tree was constructed using the Manhattan coefficient, which, unlike the categorical coefficient, reflects the quantitative differences in the repeat copy number and accounts for the so-called summed tandem-repeat difference (STRD) (Tanner et al. 2010). The interpretation of MLVA7 was according to previously established criteria (Tanner et al. 2010).

RESULTS AND DISCUSSION

Duplex EvaGreen real-time PCR validation

The simultaneous identification of *gluD* and *tcdB* in a single duplex real-time PCR was based on the difference between the melting temperatures (Tm) of the amplicons (*gluD* Tm = 81 °C; *tcdB* Tm = 79 °C) (Fig. 1a, b). No false positives or false negatives were registered when testing the specificity panel, providing 100% analytical specificity (data not shown). The estimated analytical sensitivity was 24 genome equivalents per reaction for *gluD* and 2.4 genome equivalents for *tcdB* (Fig. 1c).

*C. difficile* identification

The enzyme immunoassay A/B test resulted in 33 positive samples all of which were also culture positive. In total, 40 isolates were recovered by stool culture, from which 32 were PCR positive for *gluD* and were confirmed as *C. difficile*. The latex agglutination test appeared to be less specific, identifying 38/40 isolates as *C. difficile*. The six repeatedly false-positive isolates in the Culturette latex agglutination test were excluded from further investigations as they were confirmed with another latex kit (*C. difficile* Test kit; Oxoid) and were negative in toxin-detecting PCRs, ribotyping and MLVA. The most plausible explanation for the false-positive results is probably cross-reactions with antigenically closely related species (Huovinen et al., 1990), although the manufacturer of the CCFA (Hi-Media) states that it should suppress the growth of non-*difficile* clostridia, and our quality control tests confirmed this (data not shown).

Toxin detection

Twenty-nine out of the 32 *C. difficile* isolates were positive for both *tcdA* and *tcdB* with PCR, with the remaining three negative for both genes. Despite the doubtful clinical relevance of these non-toxigenic strains, we included them in the study as the stool specimens were A/B positive by enzyme immunnoassay, the patients responded well to *C. difficile*-specific therapy and no other compatible aetiology was documented. The intact *tcdA* gene was detected in 20 of the *tcdA*-positive isolates whereas the remaining nine harboured the truncated gene with a specific deletion (1.82 kb) and expressed an inactive toxin (Kato et al., 1999).

Three of the A+B+ *C. difficile* isolates additionally produced a binary toxin (CDT), whilst the A-B+ isolates were all CDT negative. Previous studies have shown that the prevalence of *cdtA* and *cdtB* genes in human *C. difficile* isolates from Europe varies from 1.6 to 20.8%, which is in agreement with our data (Rupnik et al., 2003, 2005). In summary, four toxigenic profiles were distinguished in this study: A+B+ CDT− (53.1%, 17/32), A−B+CDT− (28.1%, 9/32), A+B+CDT+ (9.4%, 3/32) and A−B−CDT− (9.4%, 3/32).

Typing and subtyping

PCR ribotyping was used for further characterization of the 32 clinical *C. difficile* isolates. In contrast to agarose gel electrophoresis currently used in most laboratories worldwide, we analysed intergenic spacer region (ISR) PCR products by non-sequencer-based Q-CGE. Recently, this system was evaluated in comparison with sequencer-based CGE (Xiao et al. 2012). The authors concluded that Q-CGE has several disadvantages, mainly the low intensity of the DNA fragments with size >500 bp and the inability to discriminate between fragments whose sizes differ by less than 2 bp. Initially, we faced the same problems with the sensitivity of the larger fragments. Variations in different PCR parameters were tested in an attempt to decrease the preferential amplification of the shorter fragments. We found that using an alternative PCR master mix specifically designed for multiplex applications (Quantitect Multiplex PCR kit; Qiagen) in most cases alleviated the PCR competition in ribotyping (data not shown). Q-CGE was definitely more informative than agarose gel electrophoresis and, despite its lower discriminatory power compared with sequencer-based CGE, we strongly recommend using this method in low-resource settings for ribotyping.

Fig. 1. (a) Duplex EvaGreen real-time PCR of the *gluD* and *tcdB* genes. (b) Melting curves of the *gluD* and *tcdB* amplicons for the clinical strains (●), positive-control *C. difficile* ribotypes 002 and 027 from the 25 *C. difficile* strains collection from the ECDC (■), negative control of ddH2O (▲). RFU, relative fluorescence units. *gluD* had a Tm of 81 °C and *tcdB* had a Tm of 79 °C. (c) Sensitivity experiment of duplex PCR for *gluD* and *tcdB*. Lanes: 1, 2.4×10⁶ c.f.u. per reaction; 2, 2.4×10⁸ c.f.u. per reaction; 3, 2.4×10⁹ c.f.u. per reaction; 4, 2.4×10⁵ c.f.u. per reaction; 5, 2.4×10⁸ c.f.u. per reaction; 6, 2.4 c.f.u. per reaction; 7, 2.4×10⁻¹ c.f.u. per reaction; 8, 2.4×10⁻² c.f.u. per reaction; 9, negative control of dd H2O; 10, DNA marker, 50–500 bp. Right and left numbers represent DNA fragment sizes in base pairs.
Fig. 2. MLVA7 subtyping of clinical and reference (RS) *C. difficile* isolates. Non-amplifiable loci are indicated by a 0 repeat copy number (blue boxes). The red boxes show clusters of isolates belonging to ribotype 014/020 and ribotype 017. The categorical coefficient and UPGMA methods were used for clustering and construction of the dendrogram. GT, genotype; HC, hospital code.
The most prevalent ribotype in this study was 017 (28.1 %, 9/32). These results correspond well with the findings in many other countries (Poland, Korea and Japan), which also found 017 as the leading C. difficile ribotype (Pituch et al. 2006). PCR ribotype 014/020 (15.6 %, 5/32) ranked second after 017. The remaining ribotypes, 001, 002, 012, 023, 046, 070 and 078, were detected sporadically and were represented by up to two isolates. The ribotype profiles of six isolates, one of which was toxin A/B negative, differed from each other and from the 25 reference C. difficile isolates provided by the ECDC. In addition, the banding patterns of two toxin A/B-negative strains from the same hospital were very similar to 046 and were designated 046-like. It has been estimated that >300 distinct ribotypes have been identified so far and yet there is no public reference ribotype database, which hampers the establishment of internationally recognized nomenclature.

All the Bulgarian isolates from ribotype 017 were positive for tcdB (A\(^+\)B\(^+\)CDT\(^−\)) and were detected in four hospitals in Sofia. Four patients >75 years old diagnosed with C. difficile ribotype 017 died, and two were from the same hospital. Reports from other European authors have also shown a high prevalence of severe forms of CDI caused by the hypervirulent PCR ribotype 017 (Barbut et al. 2007). A possible explanation for this could be the increased survival rates of the organism in the hospital environment due to a high sporulation frequency and resistance to disinfectants (Dawson et al. 2011). In addition, ribotype 017 has been associated with a high level of resistance to clindamycin and erythromycin (Pituch & Brazier et al. 2006).

Isolates of the 014/020 ribotype were found to be A\(^+\)B\(^+\)CDT\(^−\) and originated from three hospitals in Sofia and one in Plovdiv. Interestingly, only relatively young adults (mean 32.4 years) were affected by this ribotype. The 014/020 ribotype is considered to be present ubiquitously in the environment and has been isolated from humans, animals and various foods (Janicic et al. 2012). Similar to the findings in this study, ribotype 014 and 020 banding patterns have often been found to be indistinguishable (Janicic et al. 2012). However, recent data indicate that they are otherwise genetically unrelated and should not be grouped together (Marsden et al. 2010).

A single isolate typed as 078 was recovered from a severely ill 66-year-old patient who subsequently died. As previously reported, 078 ribotype is generally characterized by the presence of all three toxins (A\(^+\)B\(^+\)CDT\(^+\)) and these features, along with others, contribute to the more severe CDI course and higher fatality rate (Baldan et al. 2010).

The MLVA7 method was used for subtyping of all 32 clinical and a subset of reference strains. A total of 36 MLVA7 genotypes were detected: 24 for the clinical and 12 for the reference C. difficile isolates. In two instances, different colonies from a single specimen resulted in MLVA7 profiles differing by up to two STRD, a phenomenon reported previously by others (Tanner et al. 2010) and reflecting the insufficient stability of the variable-number tandem repeats (VNTRs) in the current MLVA scheme. The A6 VNTR could not be amplified in all ribotype 78, one ribotype 023 and one ribotype 017 isolates, even at an annealing temperature that was 5 °C lower (data not shown). These problems have been reported previously and are associated with primer mismatches and even the apparent absence of A6 from ribotype 078 genomes (Bakker et al. 2010).

Interestingly, ribotype 017 and 014/020 isolates formed distinct clusters, which might be indicative of clonal spread among certain hospitals (Figs 2 and 3). The clinical ribotype 017 isolates were grouped into five MLVA7 types designated 4, 18, 19, 23 and 25, whereas ribotype 014/020 was subtyped into two MLVA types, 27 and 32 (Fig. 2).
In this study, MLVA7 showed a relatively good correlation with ribotyping, although novel MLVA schemes providing greater concordance with ribotyping are needed and some progress has already been made in this direction (Wei et al. 2011).

To conclude, the majority of *C. difficile* isolates in Sofia and Plovdiv regions belonged to outbreak ribotypes 017 and 014/20, which are widely distributed in Europe. The innovative duplex EvaGreen real-time PCR protocol for simultaneous detection of the *gluD* and *tcdB* genes proved to be highly effective for confirmation of clinical *C. difficile* isolates. As the majority of pathogenic *C. difficile* isolates harbour the *tcdB* gene, the method could be validated further as a complementary diagnostic tool for direct detection in clinical specimens, and our preliminary data with 50 stool samples are strongly supportive of its potential. The MLVA7 genotypes detected in this study correlated well with the discovered ribotypes and allowed the establishment of two clonal complexes.

The results of the present study support strongly the diagnostic and therapeutic preparedness of Bulgarian hospitals for CDI. However, an important limitation of this study is that the isolate set originated only from two cities and is not representative of the national incidence rate as well as the general diversity of *C. difficile*. Nevertheless it is an important first step towards improving the surveillance of CDI in Bulgaria.

**ACKNOWLEDGEMENTS**

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**REFERENCES**


