Use of different molecular typing methods for the study of heterogeneity within *Clostridium difficile* toxinotypes V and III

S. Janezic,1 A. Indra,2 F. Allerberger2 and M. Rupnik1,3

1Institute of Public Health Maribor, Centre for Microbiology, Prvomajska 1, 2000 Maribor, Slovenia
2Austrian Agency for Health and Food Safety (AGES), Institute for Medical Microbiology and Hygiene, Waehringerstraße 25a, 1090 Vienna, Austria
3Faculty of Medicine, University of Maribor, Slomskov trg 15, 2000 Maribor, Slovenia

*Clostridium difficile* strains of toxinotypes III (n=13) and V (n=45) were typed by agarose gel-based PCR ribotyping, capillary gel electrophoresis-based PCR ribotyping and PFGE using two different restriction enzymes, *SmaI* and *SacI*. With conventional agarose gel-based PCR ribotyping, toxinotype III strains were distributed among six different PCR ribotypes and toxinotype V strains into three different PCR ribotypes. Capillary gel electrophoresis-based ribotyping was more discriminatory for toxinotype V strains, with six different ribotypes found. With PFGE using *SmaI*, all toxinotype III strains grouped together into a single pulsotype. Using *SacI*, ribotype 027 strains grouped together with >90% similarity and were <83% similar to other ribotypes of toxinotype III strains. Within ribotype 078, seven (*SmaI*) and eight (*SacI*) different pulsotypes were found, whilst ribotype 126 strains belonged to one (*SmaI*) and two (*SacI*) pulsotypes. Within ribotype 066, it was possible to distinguish between pig and human isolates. Using *SacI*, a further distinction could also be made between pig isolates from two different farms. PFGE (*SmaI* and *SacI*) clustered strains according to their toxinotype; however, correlation of PFGE and ribotyping was better with *SacI*. These data suggest that toxinotype III strains are a more heterogeneous group than toxinotype V strains and that *SacI* is more discriminatory than *SmaI*. Alternatively, the use of both enzymes simultaneously could improve PFGE typing of *C. difficile*.

**INTRODUCTION**

*Clostridium difficile*, the causative agent of antimicrobial-associated diarrhoea and pseudomembranous colitis, is currently one of the most important nosocomial pathogens (Rupnik et al., 2009). Furthermore, the incidence of community-acquired *C. difficile* infections has increased in recent years and *C. difficile* has also emerged as a pathogen or commensal in domestic animals such as pigs, calves and chickens (Gould & Limbago, 2010; Weese, 2010).

Several *C. difficile* genotypes currently predominate in the human population. PCR ribotype 027/toxinotype III (BI/NAP1/027) has gained much attention as a hypervirulent type associated with increased mortality and disease severity and has spread in North America and Europe since 2003 (Kuijper et al., 2006). It is now slowly being replaced by other genotypes, e.g. type 106 in the UK. One of the newly emerging types associated with severe disease is PCR ribotype 078/toxinotype V (Baldan et al., 2010; Goorhuis et al., 2008). Interestingly, this type has typically been associated with animal hosts (Keel et al., 2007; Rodriguez-Palacios et al., 2006) and was rarely found in humans. Lately, however, a substantial increase in its prevalence in humans has been reported (Bauer et al., 2011; Goorhuis et al., 2008; Jhung et al., 2008; Limbago et al., 2009; Rupnik et al., 2008).

Toxinotypes III and V can include other PCR ribotypes in addition to 027 and 078 (Rupnik, 2008). Together with toxinotype VIII (ribotype 017), they belong to the three most prevalent groups of variant strains (Barbut et al., 2007; Bauer et al., 2011).

To understand better the epidemiology and possible transmission routes in hospitals and community and animal reservoirs, several molecular typing methods are widely used, among which PFGE and PCR ribotyping are considered the gold standards (Rupnik et al., 2009). Some new methods such as multilocus variable-number tandem repeat analysis (MLVA) can further differentiate strains within pulsotype or PCR ribotype (Bakker et al., 2010; Fawley et al., 2008; Killgore et al., 2008). Toxinotyping is less discriminatory than PFGE and PCR ribotyping but gives information on changes in the toxin-coding.

Abbreviations: MLVA, multilocus variable-number tandem repeat analysis; PaLoc, pathogenicity locus.
pathogenicity locus (PaLoc) region (Rupnik, 2008). For phylogenetic studies of evolution within important lineages (ribotypes), comparative genomics is used applying either microarray approaches (Stabler et al., 2006, 2009) or genome sequence analysis (He et al., 2010).

Here, we describe a comparison of three different typing methods for study of the variability within two currently important C. difficile toxinotypes, III and V.

METHODS

**C. difficile isolates.** A total of 63 C. difficile isolates of toxinotypes III (n = 13), V (n = 45), VI (n = 4) and VII (n = 1) from different geographical regions and time periods were studied. Eight toxinotype V strains were from animals: six were from piglets from two different Slovenian farms, one was from a horse from the USA and one was from a calf from Canada. The remaining 55 isolates were from humans and originated from Austria, Belgium, Japan, Ireland, Germany, the USA, Denmark, the UK, France and Canada. Strains of toxinotypes VI and VII were included as they are very similar to toxinotype V in the PaLoc region, differing only in tcdA deletions. In addition, toxinotypes VI and VII contain the same ribotypes as toxinotype V.

**Molecular characterization.** All isolates were characterized previously by toxinotyping involving amplification and enzymatic restriction of PCR fragment A3 of tcdA and PCR fragment B1 of tcdB (Rupnik et al., 1998). Binary toxin genes as described by Stubbs et al. (2000).

**Agarose gel-based PCR ribotyping.** Agarose gel-based PCR ribotyping was performed with primers 16S (5'TGCGGCTGGATCACCTCCT-3') and 23S (5'CCGTACCCCTAAATAACTTGACC-3'), as described by Bidet et al. (1999). PCR products were concentrated to a final volume of 25 μl by heating at 75 °C for 45 min before electrophoresis in a 3% agarose gel (Bio-Rad) for 5 h at 2.5 V cm⁻¹. BioNumerics software version 6.10 (Applied Maths) was used to analyse banding patterns. PCR ribotypes for which reference strains were available were designated by standard Cardiff nomenclature (027, 066, etc.), whilst others were designated by internal nomenclature (SLO and a 3-digit code).

**Capillary gel electrophoresis-based ribotyping.** Capillary gel electrophoresis-based ribotyping was performed as described elsewhere (Indra et al., 2008). PCR fragments were analysed using a 310 Genetic Analyzer (Applied Biosystems) with a 41 cm capillary loaded with a POP4 gel. A 50–625 bp TAMRA ladder (Chimerx) was used as an internal marker for each sample. Injection of samples was carried out at 5 kV over 5 s, with a total running time of 28 min at a 15 kV run voltage. The size of each peak was determined using Peakscanner software version 1.0 (Applied Biosystems). Peaks were counted as bands when they showed at least 10% of the height of the highest peak of the individual run. Double peaks were counted only if they were separated by more than 1.5 bp. PCR ribotypes were identified using the web-based software program AGES-WEBRIBO (http://webribo.ages.at).

**PFGE.** PFGE was performed as described elsewhere (Janezic & Rupnik, 2010). Genomic DNA was digested with 15 U SacII or Smal overnight and a Biometra PFGE system was used for electrophoresis. Dendrograms were constructed using BioNumerics software version 6.10 by the UPGMA clustering method, using the Dice coefficient with position tolerance and optimization of 1.10%. Clusters with $\geq 80\%$ (Smal) or $\geq 85\%$ (SacII) similarity were considered to be distinct pulsotypes. Within each type, clusters with $\geq 95\%$ similarity were considered to be distinct subtypes.

**Statistical analysis.** A $\kappa$ statistic was calculated to assess the agreement between agarose gel-based ribotyping and capillary sequence-based ribotyping. The measure of agreement was scaled from 0 (when agreement is expected to be observed by chance) to 1 (perfect agreement).

**RESULTS**

Overall, 63 isolates of C. difficile were typed with conventional agarose gel-based PCR ribotyping, capillary gel electrophoresis-based PCR ribotyping and PFGE using two different restriction endonucleases, Smal and SacII. All isolates were binary toxin-positive and belonged to four different toxinotypes, III, V, VI and VII. With all three methods, we were able to type all isolates included in the study. An overview of the typing results is shown in Table 1 and Fig. 1.

**PCR ribotyping**

With conventional agarose gel-based PCR ribotyping, 13 toxinotype III strains were distributed among six different PCR ribotypes and 45 toxinotype V strains among three different PCR ribotypes. Additionally, five strains of toxinotypes VI and VII, which are related to toxinotype V, were typed and distributed among four different PCR ribotypes. Agreement between capillary gel electrophoresis-based ribotyping and conventional ribotyping was very good, being 91% with $\kappa=0.902$ (95% confidence interval 0.820–0.984). Capillary gel electrophoresis-based PCR ribotyping was more discriminatory for toxinotype V strains, with six different PCR ribotypes found (only three 078 and two 126 strains were typed differently with capillary gel electrophoresis-based PCR ribotyping). The differences in ribotyping profiles for ribotypes 126 and 078 obtained with classical agarose gel electrophoresis and capillary electrophoresis are presented in Fig. 2. No further discrimination of ribotypes within toxinotype III was observed with this method (Table 1).

During comparison of both PCR ribotyping methods, we detected a discrepancy in the nomenclature for ribotype 066 (agarose-based)/045 (capillary-based) (Table 1). Determination of the ribotype with the agarase-based method was carried out using the reference Cardiff strains as described by Rupnik et al. (2001), whilst determination of the ribotype with the capillary-based method was based on reference strains obtained from M. Wilcox (Leeds, UK). This exemplifies the difficulties with ribotyping nomenclature as a result of the large number of reference strains needed, which are usually distributed among different laboratories.

**PFGE**

With PFGE, toxinotype III strains (n = 13) belonged to one pulsotype using Smal and five using SacII. With SacII, all III/027 (toxinotype/ribotype) strains grouped together with $\geq 90\%$ similarity and were $< 83\%$ similar to other
non-027/III strains. In contrast, all toxinotype III strains, irrespective of their PCR ribotype, grouped together into a single pulsotype with Smal. Ribotype 027 strains (n=7) formed a separate subtype using Smal (SIP13d) with one non-027 strain (ribotype SLO 041), which had an identical banding pattern to ribotype 027 strains. With Smal, one ribotype 080 and one 075 strain had an identical banding pattern (SIP13a) using Smal and were also >95% similar with SacII (pulsotype 18a). Other ribotypes of toxinotype III were grouped according to their PCR ribotype.

Within ribotype 078 (n=23), we found seven (Smal) and eight (SacII) different pulsotypes. In contrast, ribotype 126 (n=14) strains belonged to one (Smal) and two (SacII) pulsotypes. With SacII, one strain of ribotype 229 and one of 078ecdc, as determined by capillary electrophoresis-based ribotyping (both variants of PCR ribotype 126), grouped together into a single pulsotype (pulsotype 13) and were only 79% similar to the group of other ribotype 126 strains. The same two strains grouped into the same pulsotype (SIP6) using Smal as the other 126 strains but were in a separate subtype (SIP6c).

One calf isolate of ribotype 078 belonged to the same pulsotype as the other human isolates but had a banding pattern that was only 94.7 or 92.9% similar to the other human 078 group of strains using Smal or SacII, respectively. One horse isolate of ribotype 251 (078 variant) also grouped together with human 078 strains but belonged to the separate subtype with both restriction enzymes.

Within ribotype 066 (045 as determined by capillary electrophoresis-based ribotyping) (n=8), we differentiated two Smal pulsotypes, one including human and the other animal isolates. Using SacII, we could also further distinguish between pig isolates from two different farms.

Toxinotype VI strains (ribotypes SLO 012, SLO 024 and 045) were grouped together into a single pulsotype with both restriction enzymes. Both ribotype 063 strains (toxinotypes VI and VII) had indistinguishable banding patterns and formed a separate pulsotype with SacII (pulsotype 12) and separate subtype within the 078 group of strains (SIP1d) with Smal.

### DISCUSSION

Ribotype 078/toxinotype V is a population of strains that is of interest for several reasons. It was predominant in animals in the past (Keel et al., 2007; Rodriguez-Palacios et al., 2006) and only recently has been found more often in humans (Bauer et al., 2011; Goorhuis et al., 2008; Rupnik et al., 2008). Previous studies based on comparative genomics and multilocus sequence typing have shown ribotype 078 to be a distinct lineage within C. difficile.
(Griffiths et al., 2010; He et al., 2010; Marsh et al., 2010) and that ribotype 078 diverged rather early in C. difficile evolution (He et al., 2010). In our study, toxinotype V strains were distributed by PCR ribotyping into three different ribotypes, 078, 126 and 066. Within each ribotype, strains were also very similar to each other by PFGE, regardless of their origin of isolation or geographical location. These results are in concordance with previously published data on comparison of ribotype 078 strains using different MLVA schemes (Bakker et al., 2010; Goorhuis et al., 2008; Marsh et al., 2010) and also support the previously published observation that this high level of relatedness within 078 strains could be a consequence of a less natural variability within ribotype 078 strains, as was suggested by Bakker et al. (2010). In our study, this genetic similarity was not observed for ribotype 078 strains only but also for other ribotypes of toxinotype V (126 and 066), which were even less variable with PFGE (one and two different pulsotypes for 126 and 066, respectively). Toxinotype V strains are related to some other toxinotypes (VI and VII) in the PaLoc region, showing a difference only two different pulsotypes for 126 and 066, respectively.

PFGE was one of the first methods described for analysis of C. difficile. Although labour-intensive, it is still considered the gold standard in North America. Inter-laboratory comparison of data became possible with the use of standardized protocols and computer software for analysis of banding patterns. For restriction of genomic DNA for typing of C. difficile, most groups use Smal (Killgore et al., 2008; McDonald et al., 2005). However, our data showed that the SacI restriction enzyme could be more discriminatory than Smal. For instance, pig isolates of toxinotype V/ribotype 066 from two different farms had identical banding patterns with Smal, but with SacI we were able to differentiate the isolates from these two farms (Fig. 1 and Table 1). Use of SacI for PFGE typing of C. difficile has been described previously by Kato et al. (1994, 1998); however, no difference in discriminatory power was described when compared with Smal. The criteria proposed by

Fig. 1. PFGE (SacI) dendrogram with results of agarose gel-based PCR ribotyping and PFGE using Smal. The dendrogram is colour-coded, with each colour representing a unique Smal pulsotype. For each strain, only the PCR ribotype determined by agarose gel-based PCR ribotyping is shown; only in cases where the capillary gel electrophoresis-based PCR ribotyping was more discriminatory is the latter also added in parentheses.

Conventional agarose gel-based PCR ribotyping is easy and cheap, but, as with other non-sequence-based methods, it relies on comparison of bands in agarose gels and therefore inter-laboratory comparisons of data are very difficult. Currently, reference strains are needed to assign the ribotype correctly, and distribution of strains through various laboratories is still problematic. Capillary gel electrophoresis-based PCR ribotyping, supported by a web-based database, could be an answer to overcome the problems associated with comparison of typing results between laboratories (Indra et al., 2008) and to make the ribotyping nomenclature more uniform.

Currently, there is no standard typing method for C. difficile, and different laboratories use different methods. Most of the methods used are able to cluster isolates that appear to be related. Some (e.g. multilocus sequence typing, PFGE and PCR ribotyping) are more appropriate for global comparisons, tracking the spread of specific types and phylogeny studies, whilst others with higher discriminatory powers (e.g. MLVA and restriction endonuclease analysis) are more appropriate for the investigation of local spread, particularly in outbreak situations (Killgore et al., 2008).

Similarly, ribotype 027/toxinotype III strains also form a separate clade, as has been shown by comparative genomic analysis (He et al., 2010; Stabler et al., 2009). In contrast to toxinotype V, toxinotype III strains included in the study were more heterogeneous, which is in agreement with previous studies of ribotype 027 strains with different MLVA schemes (Drudy et al., 2008; Fawley et al., 2008; Killgore et al., 2008).

Currently, there is no standard typing method for C. difficile, and different laboratories use different methods. Most of the methods used are able to cluster isolates that appear to be related. Some (e.g. multilocus sequence typing, PFGE and PCR ribotyping) are more appropriate for global comparisons, tracking the spread of specific types and phylogeny studies, whilst others with higher discriminatory powers (e.g. MLVA and restriction endonuclease analysis) are more appropriate for the investigation of local spread, particularly in outbreak situations (Killgore et al., 2008).

PFGE was one of the first methods described for analysis of C. difficile. Although labour-intensive, it is still considered the gold standard in North America. Inter-laboratory comparison of data became possible with the use of standardized protocols and computer software for analysis of banding patterns. For restriction of genomic DNA for typing of C. difficile, most groups use Smal (Killgore et al., 2008; McDonald et al., 2005). However, our data showed that the SacI restriction enzyme could be more discriminatory than Smal. For instance, pig isolates of toxinotype V/ribotype 066 from two different farms had identical banding patterns with Smal, but with SacI we were able to differentiate the isolates from these two farms (Fig. 1 and Table 1). Use of SacI for PFGE typing of C. difficile has been described previously by Kato et al. (1994, 1998); however, no difference in discriminatory power was described when compared with Smal. The criteria proposed by

Fig. 2. Comparison of PCR ribotyping patterns obtained with conventional agarose gel-based ribotyping and capillary gel electrophoresis-based ribotyping. Numbers represent the size of individual peaks obtained by capillary electrophoresis (bp).
Tenover et al. (1995), which consider two isolates as genetically unrelated if their banding pattern differs by seven or more fragments, are usually applied for analysis of PFGE data. These criteria are reliable if there are at least ten distinct fragments in the PFGE profile. The lack of discrimination for SmaI in some cases can therefore be explained by the fact that restriction of genomic DNA with SmaI gives fewer fragments (in our set of strains, there were 7–11 fragments) than restriction with SacII (11–16 fragments). For cluster analysis of PFGE data using SmaI, we previously used defined criteria, where clusters with ≥80% similarity were defined as distinct pulsotypes (Killgore et al., 2008). For SacII, taking into account the guidelines proposed by Tenover et al. (1995), we defined a new cut-off value and considered clusters with ≥85% similarity to be distinct pulsotypes.

Agreement between both ribotyping methods was very good. The results of capillary gel electrophoresis-based ribotyping and conventional ribotyping were concordant good. The results of capillary gel electrophoresis-based PCR ribotyping (Table 1 and Fig. 2), which was due to the better resolution of capillary gel electrophoresis in comparison with agarose gel electrophoresis, as was shown previously with ribotype 014 strains (Indra et al., 2008). PFGE (SmaI and SacII) clustered strains according to their toxinotype; however, correlation of PFGE and ribotyping was better with SacII (Fig. 1 and Table 1). In some instances, PCR ribotyping or toxinotyping provided greater discrimination than PFGE. Ribotypes 075 and 080 (toxinotype III) had identical banding patterns with both restriction enzymes. Two 063 strains had identical banding patterns with agarose gel-based PCR ribotyping and PFGE but were of different toxinotypes (VI and VII) and different subtypes with capillary gel electrophoresis-based ribotyping. As mentioned previously, these two toxinotypes differ only in the length of deletion in the repetitive domain of tcdA and it is possible that they have evolved from a common ancestor, which could be toxinotype V (Rupnik et al., 2001).

Toxinotype V strains have been linked to community-associated C. difficile infections (Limbago et al., 2009) and have been found in food animals (Avbersek et al., 2009; Rodriguez-Palacios et al., 2006) and food (Weese, 2010). In this study, toxinotype V/ribotype 078 strains isolated from animals and humans were similar. These results are in agreement with data from other recent publications of comparisons of human and animal ribotype 078 isolates using MLVA (Bakker et al., 2010; Goorhuis et al., 2008) and toxinotype V using PFGE (Jung et al., 2008). Human and pig ribotype 066 isolates were more diverse with only 50–57% similarity. This could be explained by the fact that 066 strains tend to be sporadic, rarely causing disease in humans, and that the two human strains were isolated in 1995 and the pig strains 10 years later.

Our data suggest that toxinotype III strains are a more heterogeneous group than toxinotype V strains. Due to the better discrimination observed for SacII and better correlation with PCR ribotyping, SacII could be substituted for SmaI, or both enzymes could be used simultaneously, to improve PFGE typing of C. difficile.

ACKNOWLEDGEMENTS

S.I. was supported by the Slovenian Research Agency, grant 1000-08-310144. M.R. and A.I. were supported by ERA NET Pathogenomics project CDIFFGEN.

REFERENCES


Typing of C. difficile toxinotypes III and V


