Investigation of *Clostridium difficile* interspecies relatedness using multilocus sequence typing, multilocus variable-number tandem-repeat analysis and antimicrobial susceptibility testing

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**ABSTRACT**

Multilocus sequence typing (MLST), multilocus variable-number tandem-repeat analysis (MLVA) and antimicrobial susceptibility were performed on 37 animal and human *C. difficile* isolates belonging to 15 different PCR-ribotypes in order to investigate the relatedness of human and animal isolates and to identify possible transmission routes.

MLVA identified a total of 21 different types while MLST only distinguished 12 types. Identical *C. difficile* strains were detected in the same animal species for PCR-ribotypes 014, 078, UCL 16U and UCL 36, irrespective of their origin or the isolation date. Non-clonal strains were found among different hosts; however, a high genetic association between pig and cattle isolates belonging to PCR-ribotype 078 was revealed. MLVA also showed genetic differences that clearly distinguished human from animal strains. For a given PCR-ribotype, human and animal strains presented a similar susceptibility to the antimicrobials tested. All strains were susceptible to vancomycin, metronidazole, chloramphenicol and rifampicin, while PCR-ribotypes 078, UCL 5a, UCL 36 and UCL 103 were associated with erythromycin resistance.

The data suggest a wide dissemination of clones at hospitals and breeding-farms or a contamination at the slaughterhouse, but less probability of interspecies transmission. However, further highly discriminatory genotyping methods are necessary to elucidate interspecies and zoonotic transmission of *C. difficile*.

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**Introduction**

*Clostridium difficile* is an anaerobic spore-forming bacterium responsible for serious enterocolitis in humans and animals. Several outbreaks have been reported in recent years, particularly in hospitals, making *C. difficile* a primarily nosocomial pathogen in humans (Jones et al., 2013). However, the increasing number of *C. difficile* infections (CDI) acquired in the community (Hensgens et al., 2012) and the large number of reports describing food (Rodriguez-Palacios et al., 2013), animal (Susick et al., 2012) and environmental (Zidaric et al., 2010; Hargreaves et al., 2013) reservoirs of the bacterium suggest possible transmission outside healthcare settings. For this reason, several studies have considered food and animals as potential sources for human community-acquired CDI (Rodriguez-Palacios et al., 2013).

Previous studies have focused on comparing *C. difficile* isolates from diverse sources and hosts (Lemée et al., 2005; Bakker et al., 2010; Griffiths et al., 2010; Stabler et al., 2012). Multilocus variable-number tandem-repeat analysis (MLVA) and multilocus sequence typing (MLST) have been recently developed to bring out phylogenetic relationships among the *C. difficile* population. MLVA shows a high level of discrimination and is considered useful for tracking outbreak strains geographically and for identifying cross-infection clusters between patients (Killgore et al., 2008; Manzoor et al., 2011). Data obtained by MLST can be used to determine the molecular phylogeny of *C. difficile* isolates and are highly transportable inter-laboratory (Killgore et al., 2008).

Few studies using MLVA and MLST methods have focused on comparing human and animal isolates. Three previous studies (Lemée et al., 2004, 2005; Stabler et al., 2012) analysed isolates from various hosts by MLST, and the MLVA technique has been applied to determine the relatedness of *C. difficile* PCR ribotype 078 and 027 isolates from different hosts (Debast et al., 2009; Bakker et al., 2010).

In this study MLVA and MLST were both used to investigate genetic relationships between *C. difficile* isolates from human faecal
samples, pig and cattle intestinal and carcasses samples isolated in the same geographic region and during the same time period. Additionally, all of the isolates were characterised and compared with respect to their antibiotic resistance.

Materials and methods

Isolates

A total of 37 isolates were investigated. Eleven human isolates were obtained from the C. difficile collection at the Microbiology Unit, Catholic University of Louvain (the human C. difficile reference laboratory in Belgium). Another two human isolates were obtained from aged residents at a local Belgian nursing home. In animals, a total of 24 isolates were analysed; 12 were obtained from animal intestinal samples at slaughter (four from pigs and eight from cattle). The remaining 12 isolates were recovered from carcasses at the slaughterhouse (six from pigs and six from cattle). Isolates were first characterised using PCR-ribotype and toxin gene profiles using the multiplex PCR and Genotype CDiff systems (Rodriguez et al., 2012, 2013) (Table 1).

MLVA

All of the C. difficile intestinal and carcass isolates from pigs and cattle were analysed by MLVA. In addition, three isolates from hospital patients (PCR-ribotype 078) and two isolates from nursing home residents (PCR-ribotype UCL 36) were further typed by MLVA in order to compare them with the same PCR-ribotype found in animals. These human isolates were selected on the basis of their prevalence in Belgian healthcare settings.

DNA extraction was performed using a Chelex 100 Solution 5% (BioRad) as described previously (O’Neill et al., 1996). A6, B7, C5, E7, G8, CDR5 and CDR60 variable-number tandem-repeat (VNTR) loci were amplified as previously described with minor modifications (Manzoor et al., 2011). Briefly, three separate PCR duplexes (A6-B7; C6-E7; CDR5-CDR60) and one single PCR (G8) were performed. Forward PCR primers for loci CDR60, E7 and B7 were labelled with hexa-chlorofluorescein (FAM) while the remaining loci (A6, C5, G8 and CDR5) were labelled with 6-carboxyfluorescein (HEX). PCR fragments were analysed using multi-coloured capillary electrophoresis on an ABI3130 automatic sequencer (Applied Biosystems). Electropherograms were analysed using Genemapper V4.0 software (Life Technologies) and copy numbers were determined. The summed absolute difference between two MLVA-typed isolates was the calculated summed tandem repeat difference (STRD) at all seven loci were determined. The summed absolute difference between two MLVA-typed isolates was the calculated summed tandem repeat difference (STRD) at all seven loci.
Results

C. difficile MLVA analysis

A total of 26 C. difficile isolates were typed with MLVA. For PCR-ribotypes 078 and UCL 36, the animal and carcass isolates were compared with the human isolates circulating in Belgian hospitals and nursing homes. No amplification was obtained in loci A6, CDR5 and B7 for strains belonging to PCR-ribotypes UCL5a, UCL11 and UCL 078 isolated from pig and cattle intestinal contents and carcasses. In contrast, the three human PCR-ribotypes 078 were positive for both loci A6 and B7 with variable results in locus A6 and two amplifications in locus B7. For the PCR-ribotype UCL 036, although the two human isolates were different from the pig carcass strains they were similar to each other with a STRD ≤ 2. All animal isolates belonging to the same PCR-ribotype (078, 014, UCL 16U or UCL 36) obtained from the same animal species (pig or cow) and from the same type of sample (intestinal contents or carcasses) were cloned by MLVA (STRD ≤ 2). Furthermore, cloned animal isolates were detected not only within the same sampling day but also among strains collected in different dates (Table 2).

The large number of MLVA clusters identified in this study is probably related with the allelic diversity observed among the MLVA loci. The highest number of different repeats in the sample set was found for loci C6 (n = 24) and B6 (n = 15). In contrast, loci CDR5 and CDR60 presented the lowest allelic diversity. However, these loci were valuable to discriminate isolates PCR-ribotype 023, UCL 16u and 081 (CDR5), and isolates PCR-ribotype 015, 081, 023, UCL 118 and UCL 254 (CDR60) (Table 3).

C. difficile MLST analysis

A total of 12 different STs were found by MLST. ST assignment was not possible for two isolates (PCR-ribotypes UCL 254 and 014) as no loci sequence combination matched the allelic profile of the isolates. A clear concordance was found between most of the PCR-ribotypes studied and ST. However, PCR-ribotype 014 correlated with two different STs (49 and 2) while ST 11 and ST 49 were assigned to more than one different PCR-ribotype (Fig. 1). Most of the isolates belonged to clade 1, which cluster a great variety of PCR-ribotypes, including the six non-toxigenic types. Clade 5 was attributed to PCR-ribotypes 078, UCL 5a and UCL 11 whereas only one isolate (PCR-ribotype 023) was related to clade 3.

A phylogenetic tree shows a correlation between isolates with the same PCR-ribotype regardless of their origin (carcass, intestinal contents or human faeces) (Fig. 1). According to the minimum spanning tree, the nearest neighbour strains belonged to ST 49, ST 2, ST 14, ST 45 and ST 6, which corresponded with PCR-ribotypes 014, 015, UCL 16U, UCL 16L, UCL 118 and UCL 46 respectively (Fig. 2).

Discriminatory power and concordance among genotyping methods

Results of Simpson’s diversity index showed that MLVA had the greatest discriminatory power, with and index of diversity value of 0.979 (Table 4). MLVA identified a total of 21 different types, of which six grouped more than one isolate. However, most of these types (n = 15) were defined by a single isolate. MLST only distinguished 12 different STs (sequence types) generating an index of diversity value of 0.868. Wallace coefficient of concordance among genotyping methods reveals that the probability of predicting the correct ST based on knowledge of MLVA type was 71%. There were four STs that represented multiple MLVA types (comprising between two and five different MLVA types for one ST). For example, MLST could not discriminate between L, M and N MLVA groups, classified as ST15 (Fig. 1).

C. difficile antimicrobial susceptibility

All human and animal isolates were susceptible to vancomycin, metronidazole, chloramphenicol and rifampicin. In addition, all of the isolates showed full sensibility to tetracycline except for PCR-ribotypes UCL 5a and UCL 11 which both showed intermediate resistance to this drug. Only two isolates (one animal and one human strain) belonging to PCR-ribotype 078 and the two isolates belonging to PCR-ribotype UCL 5a showed in vitro resistance to trimethoprim.

Discussion

In studies of C. difficile isolates, MLVA and MLST have shown limited application to date (Griffiths et al., 2010) and few studies have investigated the phylogenetic relatedness of isolates from humans...
and from different animal species isolated in a restricted geographical region. Lemée et al. (2005) used a set of 74 C. difficile isolates from various hosts, geographic sources and PCR-ribotypes to investigate the allelic diversity and population structure of the isolates by MLST. Bakker et al. (2010) studied the relatedness of human and porcine C. difficile PCR-ribotype 078 isolates from four European countries using MLVA. In the present study, characterisation of C. difficile isolates was achieved using both MLST and MLVA methods. Six clonal groups of strains were obtained from the same animal species, irrespective of the isolation date. Furthermore, MLST revealed that all of the other human and animal isolates with a given PCR-ribotype clustered in the same lineage.

Table 3

<table>
<thead>
<tr>
<th>Simpson diversity index</th>
<th>Hunter-Gaston diversity index</th>
</tr>
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<tbody>
<tr>
<td>Locus</td>
<td>Diversity index</td>
</tr>
<tr>
<td>C6</td>
<td>0.952</td>
</tr>
<tr>
<td>B7</td>
<td>0.895</td>
</tr>
<tr>
<td>A6</td>
<td>0.885</td>
</tr>
<tr>
<td>G8</td>
<td>0.865</td>
</tr>
<tr>
<td>E7</td>
<td>0.827</td>
</tr>
<tr>
<td>CDR60</td>
<td>0.821</td>
</tr>
<tr>
<td>CDR5</td>
<td>0.814</td>
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</table>

Diversity index for VNTR data is a measure of the variation of the number of repeats at each locus (ranges from 0.0 [no diversity] to 1.0 [complete diversity]). Confidence interval: precision of the diversity index; expressed as 95% upper and lower boundaries. K: number of different repeats present at this locus in this sample set. Max (p) is the fraction of samples that have the most frequent repeat number in this locus (range 0.0–1.0).
Three of the six clonal groups of strains were identified as PCR-ribotype 078. They were detected in two hospitalised patients, in two cattle intestinal contents at slaughter and in two pigs’ intestinal contents also obtained at the slaughterhouse (specifically, in two cattle intestinal contents and in two pigs’ intestinal contents at slaughter). The human PCR-ribotype 078 clonal strains were isolated from two different Belgian provinces in two different years (unpublished data). The porcine strains were obtained on the same sampling day in one slaughterhouse; this could suggest a widespread dissemination of *C. difficile* at the slaughterhouse (Rodriguez et al., 2012) or even in the pig farm, as previously described (Keessen et al., 2011). The same conditions were observed for the two PCR-ribotype 078 isolates from cattle intestinal contents. These were considered clonal by MLVA, but in this case the isolates were obtained on two different sampling days and the animals proceeded from different herds. As previously suggested, this finding may indicate either an epidemiological connection between farms (Scheeberg et al., 2013) or slaughterhouse contamination. However, clonal strains were not detected among *C. difficile* isolates from pig and cattle origins, which indicates that cross-contamination between the two slaughter lines (bovine and porcine) during processing is unlikely.

Identical *C. difficile* strains were also detected in the same animal species for PCR-ribotypes O14, UCL 16U and UCL 36. These results indicate that clonal *C. difficile* strains are circulating among the same animal species (including humans), although interspecies transmission was not evident. In a previous study, Kenetsch et al. (2014) reported transmission between farmers and pigs but the authors did not exclude the possibility of a common environmental source of *C. difficile* for both populations. In addition, more than half of the sequenced farmer/pig pairs were not clonal. As in our study, isolates were obtained from subjects localised in different geographical regions and in different environments. It was consequently very unlikely that identical *C. difficile* isolates would be found among the different hosts although MLST and MLVA revealed a close relation between them. Furthermore, for PCR-ribotype 078 MLVA showed a higher genetic association between pig and cattle *C. difficile* isolates (STRD ≤ 6) than between animal and human isolates (STRD > 20).

Varshney et al. (2014) observed significant genotypic and phenotypic differences between meat and human isolates for a variety of PCR-ribotypes, while a few meat isolates (including PCR-ribotype 078) were very similar to human *C. difficile* strains. It has been suggested that relatedness between human and animal isolates of PCR-ribotype 078 is a consequence of less natural variability in this type than in other types (Bakker et al., 2010). However, our results indicate genetic differences that clearly distinguish between human and animal isolates.

As has been previously reported, MLST shows less discriminatory power than MLVA but does establish the *C. difficile* genetic lineage (Marsh et al., 2010). In our study some limitations of the MLVA technique were observed. Three loci, including A6, B7 and CDRS, did not seem to be stable and disappeared from some isolates, recording a null result. Variations in loci total size were also observed for some of the loci, possibly due to the weak stability of the loci in vitro after several passages which may cause isolates with closely related MLVA profiles to appear non-clonal (Wuyts et al., 2013).
Further studies to investigate the stability of these loci are therefore required. In addition, MLVA provides little insight regarding genetic relatedness. In consequence, it may be useful to combine both methods (MLVA and MLST) in order to resolve phylogenetic diversity (Zaiss et al., 2009) although the best alternative could be whole genome sequencing, which is generally considered to be the next generation tool to type bacterial strains.

Resistance of C. difficile to multiple antimicrobials has been described in several previous studies in both humans and animals (Pelaez et al., 2013; Pirset al., 2013). Even though some isolates were resistant to both moxifloxacin and erythromycin or to clindamycin and erythromycin, no association between antimicrobial resistance and toxigenic isolates was observed in our study, echoing the results of previous work by Pituch et al. (2005). Furthermore, for a given PCR-ribotype, human and animal strains presented a similar susceptibility to the antimicrobials we tested.

Our study has some limitations. Firstly, only two different human PCR-ribotypes were analysed by MLVA because the other types found in animals were either not available in the human collections in our laboratory or have not been frequently isolated in previous studies.
Conducted in Belgian hospitals or nursing homes. Additionally, the lack of sufficient reference strains in our laboratory only allowed us to identify five ribotype profiles corresponding to an international collection number while the remaining PCR-ribotypes were identified using an internal nomenclature.

Conclusions

This study shows that clonal *C. difficile* strains circulate among the same animal species or among human patients, irrespective of the geographic area and the isolation date. The typing methods used also reveal close relationships between isolates of different species, but less genetic similarity among human and animal strains. However, animal and human strains cluster in the same lineage. Our data evidence the need for highly discriminatory genotyping methods, not only to elucidate the possible transmission routes between humans and animals but also to investigate animal-to-animal transmission in herds or cross-contamination at slaughterhouses.

Conflict of interest statement

None of the authors of this paper has a financial or personal relationship with other people or organisations that could inappropriately influence or bias the content of the paper.

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