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Cuperus, T.; Wit, B.; Castelijn, G.; Hengeveld, P.; Opsteegh, M.; Giessen, J.V.; ... ; Smits, W.K.

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Clostridioides difficile in calves, cattle and humans from Dutch dairy farms: Predominance of PCR ribotype 695 (clade 5, sequence type 11) in cattle

Tryntsje Cuperus [a,*], Ben Wit [b], Greetje Castelijn [c], Paul Hengeveld [a], Marieke Opsteegh [a], Joke van der Giessen [a], Céline Harmanus [d], Joffrey van Prehn [d,e], Ed J. Kuijper [d,e], Wiep Klaas Smits [d,e]

[a] Centre for Infectious Disease Control, National Institute for Public Health and the Environment (RIVM), Bilthoven, the Netherlands
[b] Netherlands Food and Consumer Product Safety Authority (NVWA), Utrecht, the Netherlands
[c] Wageningen Food Safety Research (WFSR), Wageningen University & Research, Wageningen, the Netherlands
[d] Leiden University Center for Infectious Diseases (LUCID), Leiden University Medical Center (LUMC), Leiden, the Netherlands
[e] National Expertise Centre for Clostridiodes difficile infections at Leiden University Center for Infectious Diseases (LUCID), Leiden University Medical Centre (LUMC), Leiden, the Netherlands and Centre for Infectious Disease Control, National Institute for Public Health and the Environment (RIVM), Bilthoven, the Netherlands

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ABSTRACT

Background: Clostridioides difficile is a leading cause of infectious diarrhea in both humans and livestock. In particular, *C. difficile* strains belonging to sequence type (ST) 11 are common enteropathogens. The aim of this study was to determine the presence and genetic relatedness of *C. difficile* types in dairy cattle and calves.
Method: Dutch dairy farms were visited between February and December 2021. Feces was collected from adult dairy cattle and calves of two age categories (<4 weeks and 4 weeks-4 months). Fecal samples were also requested from dairy farmers, family members and employees. Fecal samples were cultured in an enrichment medium for 10–15 days and subcultured on solid media for capillary PCR ribotyping and whole genome sequencing.
Results: C. difficile was detected on 31 out of 157 (19.8%) dairy farms. The highest prevalence was found in calves <4 weeks (17.5%). None of the 99 human samples collected were positive. Thirty-seven cultured isolates belonged to 11 different PCR ribotypes (RT) of which RT695 (56.8%) and RT078/126 (16.2%) were most abundant. In the database of the Netherlands National Expertise Centre for *C. difficile* infections (CDI, >10.000 patient isolates), RT695 was found in only two patients with hospital-onset CDI, diagnosed in 2020 and 2021. Sequence analysis of 21*C. difficile* RT695 from cattle revealed that all isolates belonged to clade 5, ST11 and contained genes encoding toxin A, toxin B and binary toxin. RT695 strains carried antimicrobial resistance genes typically found in clade 5*C. difficile*. Groups of genetically related RT695 isolates were found between dairy farms, whereas identical strains were only present in individual farms.
Conclusions: C. difficile was found in ~20% of dairy farms with a predominance of the relatively unknown RT695. Isolates of RT695 belonged to the same clade and sequence type as RT078/126, which is recognized as an important zoonotic type.

1. Introduction

Clostridioides difficile (formerly *Clostridium difficile*) is a Gram-positive, anaerobe, spore-forming bacterium. The bacterium is considered a leading cause of infectious diarrhea in both humans [1] and livestock [2], making *C. difficile* infection (CDI) a One Health problem [3]. *C. difficile* is a phylogenetically diverse species, that encompasses at least five CDI-associated clades and three different so called cryptic clades [4].

Disease caused by *C. difficile* is critically dependent on toxin A (TcdA) and/or toxin B (TcdB) that are generally contained in a specific genomic region called the pathogenicity locus, or PaLoc [5,6]. Some strains, most notably those associated with increased morbidity and mortality, additionally encode a third toxin, binary toxin (or CDT) [7]. Binary toxin-

* Corresponding author.
E-mail address: tryntsje.cuperus@rivm.nl (T. Cuperus).

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positive strains typically fall in clade 2 (including PCR ribotype 027) or clade 5 (including PCR ribotype 078).

In humans, antibiotic use is generally regarded as the main risk factor for development of CDI. However, community-acquired CDI, *i.e.* the development of CDI outside a healthcare setting and also often without antibiotic exposure, has been on the rise in recent times [8]. *C. difficile* is extensively studied in pigs, where the pathogen causes neonatal diarrhea, a significant problem in piglets [9]. Though less well-studied, *C. difficile* is known to be present in cattle as well and hypothesized as having a role in causing calf enteritis [10].

Significant overlap of strains of *C. difficile* isolated from humans and animals has been repeatedly demonstrated, in particular for binary toxin-positive isolates belonging to clade 5 (including PCR ribotypes 033, 045, 066, 078 and 126) [11–14]. In the Netherlands, pigs and pig farmers were colonized with identical (no SNP differences) and nearly identical (less than two SNP differences) *C. difficile* clones [12]. However, contact with livestock was not identified as a risk factor for community-acquired CDI [15] and both humans and animals may also be infected from a common (*e.g.* environmental) source. Thus, questions still remain about the sources and transmission routes of *C. difficile*.

The aim of this study, as part of a Dutch surveillance program for zoonotic pathogens in livestock, was to determine the presence and types of *C. difficile* in dairy cattle and calves on dairy farms, and to compare these isolates for genetic relatedness. In addition, human carriage of *C. difficile* was investigated by asking dairy farmers, their family members and employees to donate fecal samples.

2. Material and methods

2.1. Study design

The samples used in this study are part of a surveillance program for zoonotic pathogens in livestock [16]. In short, from a list of all Dutch dairy farms, a frequency distribution of farm size was prepared. Based on this distribution and in consultation with experts, a cut-off for minimal farm size of 50 adult dairy cattle was chosen to only include professional farms. Next, 200 farms were selected using probability sampling without replacement (*i.e.* probability of inclusion increased with farm size). Dairy farms were visited between February and December 2021. Feces was collected from healthy adult dairy cattle and, if present, two ages of calves (younger than 4 weeks; between 4 weeks and 4 months). From the stables with adult dairy cattle, four fecal samples were collected, each consisting of 12 scoops of fresh feces collected from the stable floor. From these four samples, one mixed sample was made directly (mixed feces sample). For the calves, one fecal sample consisting of 12 scoops was taken. For the fecal sample from calves younger than 4 weeks, usually housed alone, scoops were collected from a maximum of 12 calves in this age category. For the calves ages between 4 weeks and 4 months, housed in a group, 12 scoops were taken from the stable floor. The presence or absence of diarrhea in the sampled calves was noted.

In addition to the sample collection at the farm, dairy farmers, their family members and employees, aged 18 or above, were asked to participate in the human study. Multiple participants from one farm were allowed to participate. Participants were sent a study kit with material to collect a fecal sample and return this by regular mail to the RIVM. For this study, no ethical clearance was needed under the Dutch law, as it did not involve invasive measures. A declaration to this effect was obtained from the Medical Research Ethics Committee Utrecht (WAG/mb/20/013630, dated 08-04-2020). Informed consent was obtained from the human participants. For practical reasons, human samples were collected separately from farm visits, meaning a time difference of several weeks to months between cattle and human samples was possible.

2.2. C. difficile detection in animals samples

Five grams of feces was added to 45 ml *Clostridioides difficile* enrichment modified broth (CDEB MOD, Biotrading), gently homogenized, and incubated anaerobically at 37 °C for 10 days. Subsequently, 2 ml of the enrichment culture was mixed with 2 ml of absolute ethanol, homogenized and centrifuged at 3800 ×*g* for 10 min. The pellet was streaked onto *Clostridioides difficile* moxolactam norfloxacin (CDMN) agar with 7% horseblood (Biotrading, Mijdrecht, the Netherlands). Plates were incubated anaerobically at 37 °C for 2 days and were subsequently assessed for the presence of characteristic colonies. Suspected colonies (1–5 per plate) were restreaked onto Columbia sheep blood agar (Biotrading, Mijdrecht, the Netherlands) and incubated anaerobically at 37 °C for two days. Isolates were confirmed as *C. difficile* using the MALDI Biotyper® (Bruker Daltonics, Billerica (MA), USA).

2.3. C. difficile detection in human samples

One gram of frozen feces (mixed 1:1 with 20% glycerol in TSB) was mixed with 9 ml in-house prepared *Clostridioides difficile* broth with a moxolactam norfloxacin supplement (Oxoid, Basingstoke, UK). This was incubated anaerobically at 37 °C for 10–15 days. Subsequently, 10 µl of this enrichment was streaked onto CHROMID® *C. difficile* agar (bioMérieux, Marcy-l'Étoile, France). The enrichment was also subjected to an alcohol shock, in short, 2 ml enrichment broth was mixed with 2 ml absolute ethanol. After gentle mixing at room temperature for 50 min, the mixture was centrifuged at 3800 ×*g* for 10 min. Pellet material was streaked onto CHROMID® *C. difficile* agar. Plates were incubated anaerobically at 37 °C and evaluated for specific growth after 2 and 5 days. Suspected colonies were restreaked onto Columbia sheep blood agar (Oxoid, Basingstoke, UK) and incubated anaerobically at 37 °C for two days. Isolates were confirmed as *C. difficile* using the MALDI Biotyper® (Bruker Daltonics, Billerica (MA), USA).

2.4. C. difficile typing and sequencing

C. difficile strains were typed at the Netherlands National Expertise Center for *C. difficile*, hosted at the Leiden University Medical Center, according to standard procedures. In short, capillary PCR ribotyping [17] and a multiplex PCR targeting 16S, *gluD* and the toxin genes [18] were performed on purified total DNA. Whole genome sequencing was performed on an Illumina platform as previously described [19], and analysed using the *C. difficile* cgMLST v2 (core genome multi locus sequence typing) [20] and AMRFinder routines implemented in Seqsphere+ (Ridom). A minimal spanning tree (MST) was generated based on 2147 columns, with pairwise comparison ignoring missing values, on a logarithmic scale and an MST cluster distance threshold of 6. Assembled genome sequences were annotated *via* the Proksee server [21]. Regions from annotated genome sequences were extracted using Geneious R10.2.6 (Biomatters LLC), and visualized in clinker as implemented in the CAGECAT 1.0 server [22]. Figures were further prepared in Adobe Illustrator CC (26.3.1). Sequence data for this project are available through BioProject (NCBI) with the number PRJNA1034018.

3. Results

3.1. C. difficile is found in dairy cattle

The prevalence of *C. difficile* in both adult dairy cattle and calves from two different age categories was determined by selective culturing techniques. Overall, *C. difficile* was found on 19.8% of the sampled dairy farms (Table 1, Supplemental Fig. 1).

When analysed per age group, we found a decreasing farm-level prevalence with increasing age of the cattle (Table 1). *C. difficile* was found in the mixed feces samples of adult cattle on 3.8% of the farms, and the farm-level prevalence in the 4 weeks-4 months calves was

Table 1
C. difficile prevalence in cattle at the investigated dairy farms.

Category	Number of farms	Number of farms with positive culture(s) of *C. difficile*	Farm level prevalence	95% CI*
Adult dairy cattle	156	6	3.8%	1.4–8.2%
Calves 4 weeks-4 months	151	6	4.0%	1.5–8.5%
Calves <4 weeks	143	25	17.5%	11.6–24.7%
Total	157	31	19.8%	13.8%–26.8%

* CI: Confidence Interval, Clopper-Pearson.

similar (4.0%). The farm-prevalence based on calves younger than 4 weeks was significantly higher (17.5%) than the prevalence based on the other age groups ($p < 0.001$, Chi-square followed by pairwise comparisons). The presence of diarrhea in young calves was not associated with the presence of *C. difficile* (univariable logistic regression, $p = 0.185$).

Overall, *C. difficile* was detected on 31 farms, in 20 farms only in calves <4 weeks, in two farms only in calves 4 weeks-4 months, and in four only in the adult cattle. In four farms positive samples came from calves <4 weeks in combination with one of the other age categories. There was one farm with positive results for all age categories (Supplemental Table 1).

3.2. No *C. difficile* was identified in human participants from the same farms

To assess whether presence of *C. difficile*-positive dairy cattle is associated with colonization of the farmers with the same or a different type of *C. difficile*, we attempted to culture *C. difficile* from feces of human participants and collected information on the frequency of interaction with the cattle.

In total, fecal samples from 99 human participants, originating from 59 dairy farms, were tested for *C. difficile*. From these, 25 participants originated from a farm where *C. difficile* was detected in one or more of the animal samples. The average age of all participants was 49 (range 20–78 years) and 64% was male. From the participants, 66% was dairy farmer and the other participants were family members or employees. Ninety percent (90%) of the participants reported that they went into the cattle stables at least once a day and 83% reported physical contact with the cattle at least once a day. No *C. difficile* was found in the feces samples of the human participants (0.0%, 95%CI 0.0–3.7%).

Thus, in this study, we did not find evidence for transmission between cattle and people at the farm of *C. difficile*.

3.3. Toxigenic PCR ribotype 695 (ST11) is dominant in dairy cattle

To investigate the subtypes of *C. difficile* in cattle, we performed capillary PCR ribotyping and a multiplex PCR for the toxin genes on all isolates collected as part of this study. A total of 11 different PCR ribotypes were identified among the *C. difficile* isolates (Table 2, Supplemental Table 1). For one isolate (CD00231) no ribotype could be assigned using the database of the Netherlands National Expertise Center for *C. difficile*; notably, the multiplex PCR result suggested that this isolate is related to PCR ribotype 033 and 288, on the basis of a negative PCR result for the *tcdB* gene. The most prevalent was ribotype 695 (21/37 isolates, 57%), found on 19 different dairy farms. This PCR ribotype was positive in the multiplex PCR for genes encoding the clostridial toxins TcdA and TcdB, as well as binary toxin, similar to the epidemic PCR ribotypes 027 and 078. On two farms, two or three different ribotypes were found in the different animal categories (farm 4 – RT078 and RT695 and farm 25 – RT054, RT078, and RT695), indicating that multiple PCR ribotypes can circulate on a single farm. Four isolates were negative for binary toxin and we identified a single non-toxigenic *C. difficile* strain (NTCD, RT035).

In order to assign the isolates to a phylogenetic clade, we performed short read next-generation sequencing followed by multi-locus sequence typing. Most of the isolates (31/37, 84%), including those belonging to RT695, belonged to sequence type (ST) 11, which belongs to clade 5 (Table 2). The assignment of STs for other isolates was consistent with the determined ribotypes on the basis of published literature [19], including the assignment of the binary toxin negative isolates to clade 1.

A minimal spanning tree recapitulated the separation in clade 1 (ST6, 9, 16, 43, 107) and clade 5 (ST11, 161) isolates, with less genetic diversity observed within clade 5 (<295 alleles difference) compared to clade 1 (>1387 alleles difference) (Fig. 1).

Within the isolates of RT695 ($n = 21$), two groups were identified (MST cluster 1 with 13 isolates and MST cluster 4 with 2 isolates), separated by >12 alleles difference. Within these two MST clusters the isolates were genetically related (≤6 alleles difference, default in Seqsphere). Two pairs of RT695 isolates that were indistinguishable by core-genome MLST (C00027/C00029 and CD00247/CD00249; both in MST cluster 1) were isolated from one farm per pair (Fig. 1), but the geographical origin of the other genetically related isolates varied (Table 2 and Fig. 1).

Finally, we noted an MST cluster (MST cluster 2) with 5 isolates from ribotypes 078 and 126 (Fig. 1).

3.4. ST11 contains isolates lacking functional large clostridial toxins

The MST analysis (Fig. 1) confirmed the genetic relatedness of one RT033 isolate, an RT288 isolate, and the isolate with the unknown ribotype (MST cluster 3, these are from 3 different farms), which was suspected on the basis of the multiplex PCR (Table 2): the RT033 and the RT288 isolate were indistinguishable on the basis of cgMLST, and the unknown ribotype differed by only one allele.

As RT033 has been described to contain a large deletion in the pathogenicity locus [23], we investigated the PaLoc composition of RT033 and RT288 isolates and the isolate with no RT assigned. When raw reads from these strains were aligned to the RT078/ST11 reference genome M120 (NC_017174; [24]), we noted absence of a ~ 51 kb region that spans a region upstream of the PaLoc (from CDM120_RS03790) up into the *tcdA* (CDM120_RS04005) gene (data not shown). This is also recapitulated in an alignment of the *de novo* assembled genome of CD00222 (RT033) with M120 (Fig. 2); the positive signal for *tcdA* in the multiplex PCR is explained by the presence of a *tcdA* pseudogene that encompasses the region targeted by the PCR. The genomic context of the PaLoc remnant was highly similar for both RT033 isolates, the RT288 isolate and the isolate of unknown ribotype (data not shown); it was recovered as a single contig for strain CD00222, and as two contigs for the other strains in a *de novo* assembly, with evidence of a mobile element in the region of the PaLoc deletion (Fig. 2).

Together, these results suggest that RT033, RT288 and the unknown ribotype form a non-toxigenic cluster within ST11 as a result of a large chromosomal deletion and insertion of a mobile element.

3.5. RT695 carries less antimicrobial resistance genes than other ST11 isolates

To check whether RT695 isolates showed an antimicrobial resistance gene profile distinct from other isolates, an *in silico* analysis using AMRFinderPlus was performed. This revealed the presence of different antimicrobial resistance genes in the *C. difficile* isolates from dairy farms (Supplementary Table 1). All five clade 1 isolates were found to carry the inducible beta-lactamase gene *blaCDD* [25,26]. All clade 5 isolates carried mutations in the *gyrB* gene (S366V, S416A) which are associated with resistance to ciprofloxacin. Two clade 5 isolates carried a mutation

Table 2
Typing information of *C. difficile* isolates from cattle feces, based on capillary PCR ribotyping, PCR for toxin genes and WGS.

Farm ID	Isolate	Farm location (province)	PCR ribotype[a]	ST[b]	Clade/CC	Toxin A[C]	Toxin B[c]	Binary[c]
1	C00026	Groningen	695	11	5	+	+	+
2	C00027	Drenthe	695	11	5	+	+	+
2	C00029	Drenthe	695	11	5	+	+	+
3	C00028	Gelderland	050	16	1	+	+	−
4	CD00223	Zuid-Holland	695	11	5	+	+	+
4	CD00221	Zuid-Holland	078	11	5	+	+	+
5	CD00222	Noord-Holland	033	11	5	+	−	+
6	CD00224	Friesland	695	11	5	+	+	+
7	CD00225	Gelderland	695	11	5	+	+	+
8	CD00226	Friesland	695	11	5	+	+	+
9	CD00227	Noord-Holland	035	107	1	−	−	−
10	CD00228	Noord-Holland	005	6	1	+	+	−
11	CD00229	Zuid-Holland	126	11	5	+	+	+
11	CD00230	Zuid-Holland	126	11	5	+	+	+
12	CD00231	Noord-Brabant	no RT assigned	11	5	+	−	+
13	CD00232	Gelderland	081	9	1	+	+	−
14	CD00233	Zeeland	288	11	5	+	−	+
15	CD00234	Drenthe	657	161	5	+	+	+
16	CD00235	Noord-Brabant	695	11	5	+	+	+
17	CD00236	Gelderland	033	11	5	+	−	+
18	CD00237	Zuid-Holland	695	11	5	+	+	+
19	CD00238	Noord-Holland	695	11	5	+	+	+
20	CD00239	Friesland	695	11	5	+	+	+
21	CD00240	Overijssel	695	11	5	+	+	+
22	CD00241	Friesland	695	11	5	+	+	+
23	CD00242	Zuid-Holland	695	11	5	+	+	+
24	CD00243	Utrecht	695	11	5	+	+	+
25	CD00244	Drenthe	054	43	1	+	+	−
25	CD00245	Drenthe	078	11	5	+	+	+
25	CD00246	Drenthe	695	11	5	+	+	+
26	CD00247	Friesland	695	11	5	+	+	+
26	CD00249	Friesland	695	11	5	+	+	+
27	CD00248	Drenthe	695	11	5	+	+	+
28	CD00250	Drenthe	078	11	5	+	+	+
29	CD00251	Overijssel	078	11	5	+	+	+
30	CD00252	Drenthe	695	11	5	+	+	+
31	CD00253	Zuid-Holland	695	11	5	+	+	+

[a] Fawley et al, 2015 [17].
[b] Jolley et al, 2018 [20].
[c] ECDC, 2018 [18].

in the *gyrA* gene, associated with moxifloxacin resistance; one RT078 isolate carried a *gyrA* A188S allele and one RT695 isolated carried a *gyrA* T821I allele. A subset of clade 5 isolates (8/32, 25%) carried genes related to streptomycin and streptothricin resistance, possibly on a mobile genetic element (*ant(6)-la, aadE, sat4*). Among the isolates carrying these genes there were no RT695 isolates. Another subset of clade 5 isolates (18/32, 57%) carried tetracyclin resistance genes (*tet*(M), *tet*(O), *tet*(40)). Finally, a single RT078 isolate carried a *cfr*(B) gene which may be associated with resistance to phenicols, lincosamides, pleutomutilins and streptogramin A (so called PhLOPSA antibiotics) [27,28]. The *ermB* gene, associated with macrolide-lincosamide-streptogramin antibiotics was not found in any of the isolates. We also screened for the plasmid pCD-METRO [29] which confers metronidazole resistance, but found no evidence for its presence in these isolates (data not shown). Phenotypic resistance testing was not performed.

Together, our results suggest that RT695 does not have antimicrobial resistance genes that are uncommon for ST11 isolates.

4. Discussion

In this study, fecal samples from three different age groups of dairy cattle were investigated for the presence of *C. difficile* by enrichment broth culture, followed by selective culture. The farm prevalence of *C. difficile* was significantly higher for young calves (<4 weeks, 17.5%) compared to older calves (4 weeks-4 months, 4.0%) and adult dairy cattle (3.8%). The prevalence in adult cattle is similar to a previous study in 2009/2010, in which *C. difficile* was found in samples from 1% of adult dairy cattle at Dutch slaughter houses [30]. The observed higher prevalence in younger animals has been repeatedly reported in cattle and is also well known for pigs [31–33]. Also in humans, the carriage rate is higher in young infants [34]. It is hypothesized that natural resistance in older animals is associated with an increasing diversity of the intestinal microbiota [35].

No *C. difficile* was found in fecal samples from the dairy farmers and their family members in this work. Two previous studies similarly did not report carriage of *C. difficile* in humans with professional contact to cattle (respectively dairy and beef cattle, [32,36]). This is in contrast to a study among pig farmers, where a carriage rate of up to 25% was found for people who had daily contact with pigs [37]. It is unclear which factors may have contributed to this difference as all studies report high levels of animal contact and isolation of predominantly ST11 isolates of *C. difficile* in the animals.

A total of 11 PCR ribotypes were found among the 37 isolates, with the majority being the uncommon RT695. All RT695 isolates were toxinogenic, *i.e.* they harbored genes encoding toxins A, B and binary toxin like RT027 and RT078. In the collection of the Netherlands National Expertise Center for *Clostridioides difficile,* comprising >10,000, mostly human, strains since 2004, ribotype 695 was only found in two human cases of CDI, from 2020 and 2021. Both cases were diagnosed as community-acquired CDI. No published literature about presence of RT695 in humans or animals was found. Therefore, it is at present unknown whether RT695 is an emerging ribotype or whether it was overlooked in previous Dutch studies [17].

Our study also identified multiple other isolates that fall within clade

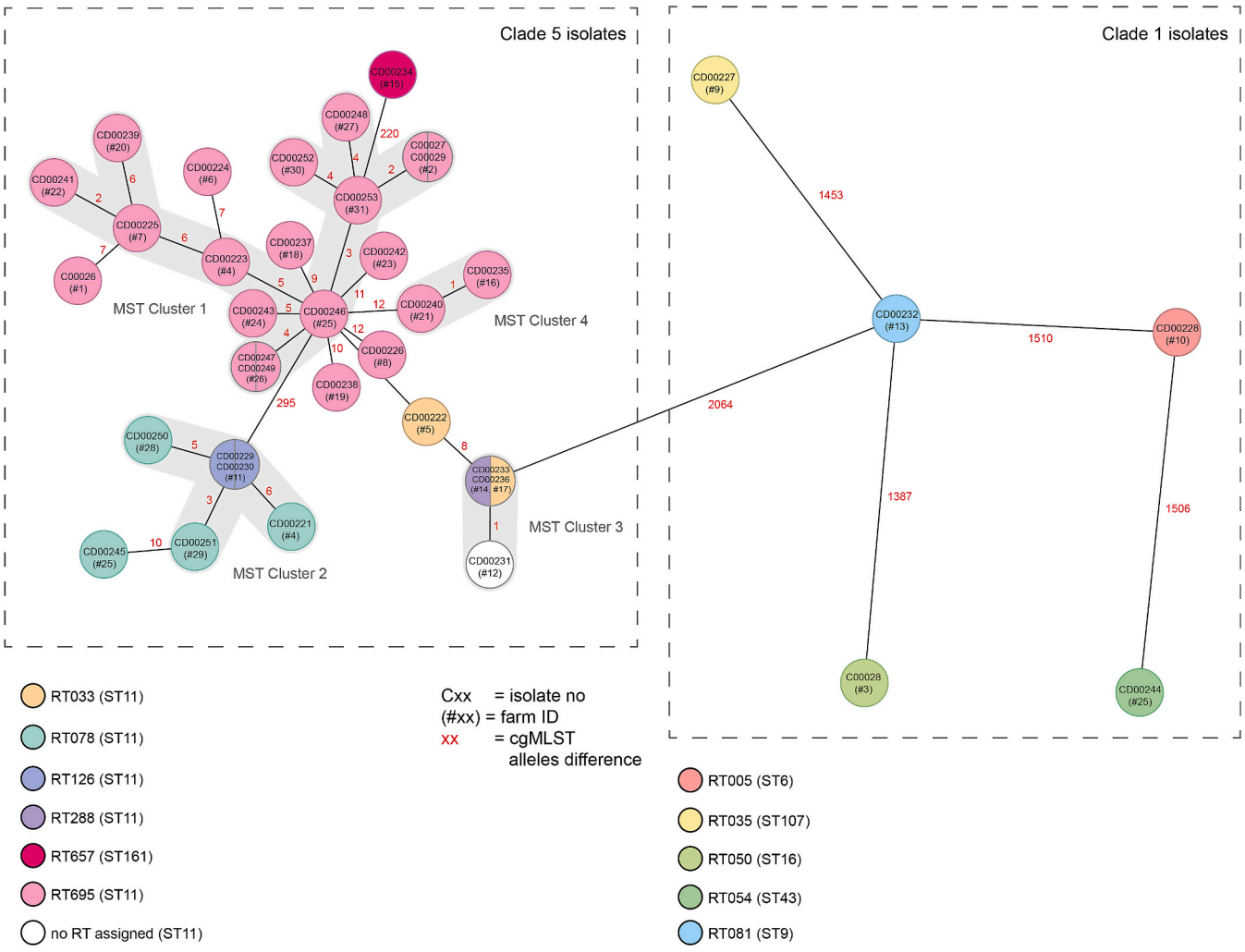

Fig. 1. Minimal spanning tree based on a core genome MLST analysis of the sequenced *C. difficile* isolates obtained from dairy cattle.

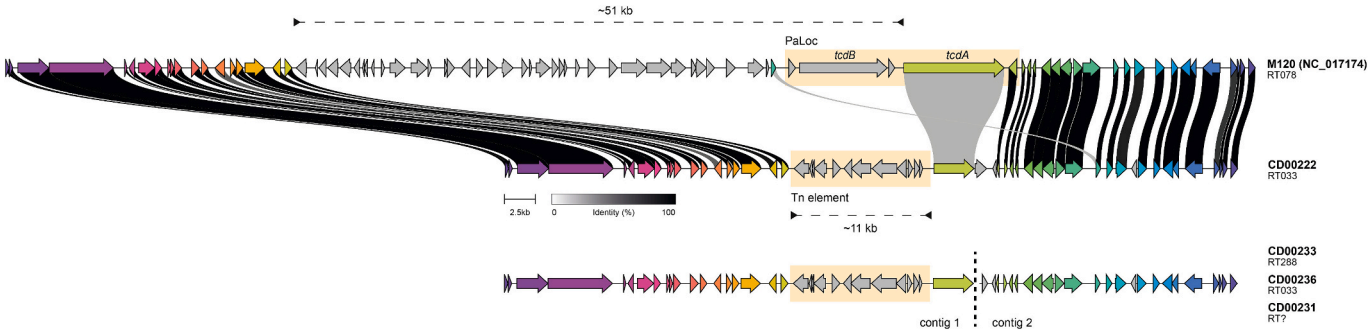

Fig. 2. Comparison of the genomic region containing *tcdA* in *tcdB*-negative ST11 isolates. Reference strain M120 contains a complete pathogenicity locus (PaLoc), whereas a large chromosomal region appears to be replaced with a putative Tn element in the RT033/RT288/RT? (unknown ribotype) isolates. Vertical dashed line indicates that for three strains this region is recovered in two contigs.

5/ST11. First, next to an unrelated RT078 isolate, a cluster (MST cluster 2) was identified that encompassed isolates belonging to RT078 and RT126 (Fig. 1), confirming the close genetic relatedness of these ribotypes [19,38,39]. Second, three other genetically related PCR ribotypes (RT033, RT288 and an unknown ribotype) that fall within ST11 were identified. Of note, in a study on *C. difficile* in calves on Slovenian dairy farms the ribotype RT033 predominated [40] and this ribotype has also been identified in Dutch calves before [30]. The fact that these ST11 isolates lack functional toxin A and toxin B genes underscores the necessity to take toxin gene identification into account when using ST designations in epidemiological studies. It is however, unclear, whether these strains should be considered non-toxigenic as previously suggested [23] as they do contain a complete CDTLoc, encoding binary toxin ([38] and this study).

In two farms, two or three different ribotypes were found in the different age categories, suggesting that multiple strains can co-exist on a farm. This was previously also shown among calves on dairy farms in the USA [36], and a study in Germany even showed individual calves

shedding more than one strain [41]. To interpret typing data for epidemiological purposes on cattle farms, it is therefore important to take multiple samples, preferably from different age groups of animals.

As expected, the *C. difficile* RT695 isolates coming from different cattle ages on the same farm were clonal, with no differences in cgMLST profile. Two clusters of RT695 isolates were found with <6 alleles difference, originating from farms across the Netherlands. Though it was previously shown that *C. difficile* isolates can be transmitted from farm to farm through trade of colonized calves [40], the economic relations between the dairy farms in our study are not known. Moreover, clade 5 isolates may show lower inter-RT allele differences than for instance clade 1 [19]. The proposed threshold for cgMLST isolates likely to belong to the same clone was previously determined at six alleles or fewer [42], though it was recently suggested that the threshold should be lowered to 3 alleles [19]. However, in the investigation involving RT078 strains, outbreak cases had 0 alleles differences. It is likely that a threshold of 0 alleles is also a better signal of epidemiological relatedness for clade 5 strains belonging to RT695, since identical strains were found within farms, but between farms all related strains did have allelic differences (1–3 alleles, Fig. 1). For these reasons, caution is warranted to interpret our results in light of possible transmission.

Finally, we note that our analysis of antimicrobial resistance genes did not show the presence of the *ermB* gene, associated with macrolide-lincosamide-streptogramin antibiotics (including erythromycin). Our data is consistent with a previous report that showed *ermB* in ~40% of human RT078 isolates, but not in animal RT078 isolates [43]. Beyond *ermB*, RT695 isolated contained a repertoire of resistance determinants that is commonly observed in other ST11 strains [32]. Future studies should evaluate the causal relationship of the identified *C. difficile* resistance genes with phenotypic resistance against relevant antibiotics.

5. Conclusion

The precise role of animals as a reservoir for human *C. difficile* infections and *vice versa* is debated. Indistinguishable or near-indistinguishable strains have been isolated from humans and different animals species, indicating potential zoonotic transmission. This study found multiple *C. difficile* ribotypes in dairy farms, with the majority belonging to clade 5, sequence type 11. RT695, an hitherto uncommon clade 5 ribotype was predominant. These results show the importance of a One Health approach to *C. difficile* surveillance, for the discovery of novel, potentially zoonotic, ribotypes and the elucidation of the reservoirs of *C. difficile*.

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CRediT authorship contribution statement

Tryntsje Cuperus: Conceptualization, Data curation, Formal analysis, Funding acquisition, Methodology, Project administration, Writing – original draft, Writing – review & editing. **Ben Wit:** Data curation, Formal analysis, Project administration, Resources, Writing – review & editing. **Greetje Castelijn:** Formal analysis, Methodology, Writing – review & editing. **Paul Hengeveld:** Data curation, Investigation, Writing – review & editing. **Marieke Opsteegh:** Conceptualization, Methodology, Project administration, Supervision, Writing – review & editing, Funding acquisition. **Joke van der Giessen:** Conceptualization, Funding acquisition, Methodology, Supervision, Writing – review & editing. **Céline Harmanus:** Investigation, Writing – review & editing. **Joffrey van Prehn:** Resources, Validation, Visualization, Writing – review & editing. **Ed J. Kuijper:** Methodology, Resources, Writing – review & editing. **Wiep Klaas Smits:** Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing, Formal analysis.

Declaration of competing interest

None declared.

Data availability

Link to data used for the research is shared in the article.

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