Clostridioides difficile in Equidae necropsied in Normandie, France, in 2019-2021: common carriage of toxigenic strains and virulence of emerging ribotype 017

Running title: *Clostridioides difficile* in French Equidae

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Abstract

Background: *Clostridioides difficile* is an anaerobic, spore-forming entero-pathogen, causing diarrhea in humans and animals. We aimed to evaluate *C. difficile* occurrence, diversity, virulence and putative circulation among French Equidae.

Methods: We took advantage of necropsies of Equidae in Normandie, France, from 2019 to 2021 (n=100), to systematically recover the caecal content and, if watery and possibly diarrheal, any intestinal content. We searched for *C. difficile* and characterized isolates for their toxin gene profile and PCR-ribotype according to the WEBRIBO database. We performed free toxin detection to reach a high-standard diagnosis of infection.

Results: Twenty-seven Equidae were positive for *C. difficile* and twenty had a toxigenic strain, including one animal also colonized by a non-toxigenic strain. Toxigenic isolates belonged to eight ribotypes: 078, 126 (three-toxin profile), 005, 012, 020, 181, AI-53 (two-toxin profile), 017 (one-toxin profile), and non-toxigenic isolates to ribotypes 009, 035 and 439. 017 and 009 were the predominant ribotypes (n=5). In two premises, few Equidae shared the same ribotype, either 020 or 009, suggesting two potential transmission clusters. Free toxin detection demonstrated *C. difficile* infection in four animals displaying *post-mortem* signs of diarrhea and toxigenic isolates of ribotype 126 (n=1) or 017 (n=3), which were the least susceptible to vancomycin. The infection by ribotype 017 caused animal death in two cases.

Conclusions: French Equidae represented an underestimated reservoir of *C. difficile* and ribotype 017 was highly virulent in these animals, both preoccupying findings in a One Health perspective.

Keywords

Clostridioides difficile, One Health, Equidae, molecular diversity, potential transmission, diagnosis, toxin detection

Introduction

C. difficile is a spore-forming anaerobe responsible for diarrhea and colitis [1]. In the 2000's, it has emerged as a major cause of outbreaks of health-care associated diarrhea in North America and Europe. Since the 2010's, it is also known as a rising cause of community-acquired infections. *C. difficile* infections (CDIs), which are transmitted by spores by the fecal-oral route, can be severe, with complications and recurrences, and life threatening. Large Clostridial Toxins (LCT) TcdA and TcdB are responsible for symptoms, even though TcdB alone is sufficient for disease, and CDT, the binary toxin, has an accessory role [2, 3]. Non-toxigenic strains are unable to cause disease, while toxigenic strains can either produce toxins and cause infection or be asymptomatically carried [4]. Antibiotics, hospitalization and age are the main risk factors for CDI. Antibiotics notably induce microbiota dysbiosis, which favors spore germination and vegetative multiplication. CDIs are treated by fidaxomicin and vancomycin, whose efficiency is decreasing after each episode [5]. Indeed, they also promote dysbiosis and therefore relapses. Spores and biofilms could contribute to persistence between relapses [6, 7]. The last resort treatment is fecal microbiota transplant (FMT) [5].

C. difficile is a One Health issue [8-10]. It can cause diarrhea in most, wild, farm and companion animals [9-13]. *C. difficile* strains can jump between hosts, leading to inter-species transmission [14-16]. Equidae are of high value (https://www.ifce.fr) and their healthcare is careful, including in hospitals. Equidae therefore share the same main risk factors for CDIs as humans. In these two monogastric hosts, *C. difficile* is responsible for outbreaks and sporadic cases [17-21]. Both have long been treated by the same antibiotics, metronidazole and vancomycin, until fidaxomicin substituted for metronidazole in the hospital, and can be treated by FMT [22]. The main difference between them as infected populations is age. While <3-years-old children are mainly asymptomatic carriers [4], CDIs can affect foals [17, 18]. Finally, Equidae, which are closely interacting with humans, are relevant to zoonotic and anthropo-zoonotic transmission.

Data about *C. difficile* importance in horses [8, 10, 11, 19] remain scarce in France. *C. difficile* testing is not systematic and in case of diarrhea, the detection of a toxigenic strain is not sufficient to diagnose a CDI [4]. Here, we studied *C. difficile* in Equidae necropsied by ANSES in Normandie, the first breeding region in France (https://www.ifce.fr). Necropsy [23] was the opportunity both to recover digestive contents from a population with diverse clinical pictures, and to get access to animal data as detailed as patient data and including the cause of death. Our objectives were to evaluate i) *C. difficile* occurrence, diversity and putative circulation among French Equidae, and ii) the prevalence of CDI or carriage after a *post-mortem* diagnosis including free toxin detection.

Materials and methods

Equine necropsy

Equine necropsies at ANSES in Normandie are performed to determine the cause of animal death, using a standardized procedure [23]. It includes examination of the cadaver, evisceration and examination of the subcutaneous connective tissue, muscles and joints, evisceration and examination of the abdominal and thoracic organs, isolation and examination of the upper respiratory tract, cervical esophagus, thyroid gland and tongue, isolation of the head and examination of the brain, guttural pouches and nasal cavities. Animal identification data, previous treatments, necropsy observations, pathogen identification, and the cause of animal death are registered according to a Thesaurus (https://sitesv2.anses.fr/fr/minisite/resumeq/pr%C3%A9sentation-de-resumeq) [23]. Here, for all animals necropsied from May 2019 to August 2021 (except fetus and stillborn foals), digestive contents were also systematically recovered in the caecum, and in case of a watery consistency, in any other intestine segment, notably the colon. All samples except one caecum were preserved as aliquots at 4°C, -20°C and -70°C. In 2018, as a pilot study, the intestinal contents of five necropsied Equidae with *post-mortem* signs of endo-enterotoxaemia and diarrhea were also collected.

Standard identification of entero-pathogens in case of infection suspicion

Escherichia coli colonies were isolated on EMB plates (Eosin Methylene Blue) and streaked on blood plates to see hemolysis. *Salmonella* spp. were identified on XLD agar plates (Thermo Scientific). *Clostridium perfringens* and *Paeniclostridium sordellii* were identified on Columbia agar + 5% sheep blood plates (BioMérieux) under anaerobiosis, and *C. difficile* was detected on ChromID (BioMérieux). Identification was confirmed by Matrix-Assisted Laser Desorption/Ionization Time-Of-Flight (MALDI-TOF) Mass Spectrometry (MS) (MBT Smart mass spectrometer, Compass and FlexControl softwares, Bruker Daltonics). Rotavirus was identified by PCR.

Specific and systematic *C. difficile* search and isolation

An aliquot of any digestive sample was directly streaked onto commercial ChromID or CLO plates (BioMérieux) and incubated for 48h at 37°C under anaerobiosis. In case of a negative result, incubation for 3-7 days in BHI broth supplemented by cysteine at 0.1%, yeast extract at 5 g/L, taurocholate at 1 g/L, D-cycloserine at 250 µg/mL and cefoxitin at 8 µg/mL (OXOID), allowed multiplication before plating onto ChromID plates. Suspicious, irregular and/or black colonies were screened by PCRs targeting *tpi*, *tcdA* and *tcdB* genes [24, 25]. Identification was finally confirmed by MALDI-TOF MS. A single colony of each confirmed strain per positive sample was grown in BHI broth at 37°C under anaerobiosis and this culture preserved in glycerol 17% at -70°C except in two cases: two initially detected strains, one non-toxigenic (animal #48) and one toxigenic (#54), which were not immediately preserved, could not be recovered again.

C. difficile characterization

All preserved isolates (N=34 from n=25 Equidae) were characterized by a multiplex PCR, revealing: i) *tpi* gene, ii) wild-type or truncated alleles of *tcdA*, *tcdB, cdtA* and *cdtB* genes and iii) the *lok* fragment characterizing non-toxigenic strains, and followed by capillary gel electrophoresis [26]. For ribotyping, PCR profiles after capillary gel-based electrophoresis [27] were analyzed with GeneMapper software (Thermo Fischer Scientific, Villebon-sur-Yvette, France) and strain ribotypes assigned according to the WEBRIBO database (https://webribo.ages.at/). The first obtained isolates had preliminary been characterized by Multi Locus Sequence Typing [28].

Susceptibility to vancomycin or metronidazole

The Minimum Inhibitory Concentrations (MICs) of vancomycin or metronidazole were determined for each isolate using Etest (bioMérieux) on Brucella blood agar (Becton Dickinson) supplemented with vitamin K1 (10 mg/L), hemin (5 mg/L) and defibrinated horse blood (5%) (Oxoid), after incubation for 24 h at 37°C.

Toxin detection

C. difficile toxins (TcdA and TcdB) and glutamate dehydrogenase (GDH) were detected in digestive contents using an Enzymatic Immuno-Assay (Quick Check Complete, Alere), according to manufacturer's instructions. Toxin detection was performed as recommended for CDI diagnosis.

Statistical analysis

Pearson's Chi-squared test or Fisher's exact test when appropriate were used. Unknown values were not imputed. Statistical significance: p-value <0.05.

Results

Necropsied Equidae

We studied the whole equine population (except fetuses and stillborn foals) necropsied in Normandie from May 2019 to August 2021, whatever animal clinical picture or death cause. After this two-years-period comprising Covid-19 and lockdowns, 100 animals had been necropsied (Table S1), a total previously reached in one year. All animals except three originated from Normandie (Table S1). Most were females (67%) and <1-year-old animals (59%), and notably foals (<6-months, 48%) (Table 1). They represented nine breeds, mostly Thoroughbred (42%), Trotters (31%) and Saddlebred horses (11%) (Table 1). Animal death causes included infections, non-infectious diseases and trauma. The two most prevalent causes were endo-enterotoxaemia (12%), an infection characterized by toxin-mediated inflammation [20] and infection by *Rhodococcus equi* (9%), common in foals [29].

C. difficile prevalence

Digestive contents of all animals were collected from up to two intestinal segments: i) the caecum (CAE) in all cases (n=100 animals), and ii) any other segment in case of watery consistency (CDL), possibly indicating diarrhea (n=30) (Table S1). All preserved digestive contents were tested for *C. difficile* presence on commercial screening plates. After screening of candidate colonies by standard PCRs [24, 25], and confirmation by MALDI-TOF, 27 animals were positive for *C. difficile*, in their two samples in nine cases (Table S1). *C. difficile* presence in Equidae was independent of animal sex, age, breed, antibiotic treatment or hospitalization, but significantly correlated to signs of diarrhea or endo-enterotoxaemia (Table 1). More *C. difficile*-positive animal than negative ones provided a watery sample and showed toxin mediated intestinal inflammation (Table 1).

Pathogenicity

The isolates of 25 Equidae were preserved and named '*Cd*E' (*C. difficile* from Equidae) (Table 2, Table S1). They form the first library of *C. difficile* strains originating from French Equidae, CloDifEqui (Table 2). The pathogenicity profile of all preserved isolates was characterized by PCR [26]. Non-toxigenic isolates (N=9) originated from seven animals, including six Thoroughbred, while toxigenic isolates (N=25), encoding one to three toxins, originated from nineteen animals of six different breeds (Table S1). One animal (#29) was co-colonized by a non-toxigenic strain (CAE) and a toxigenic one (CDL). For all other animals with two isolates, both were either non-toxigenic (animals #18 and #60) or toxigenic with the same toxin profile (#3, #32, #36, #37, #42 and #43) (Table 2).

Diversity of PCR-ribotypes

After PCR-ribotyping [27], all CloDifEqui isolates were assigned to eleven ribotypes (Table 2, Table S2) and four clades (1, 2, 4 and 5). Toxigenic isolates were of eight ribotypes: 126 and 078 (encoding all toxins), 020, 005, 012, 181 and AI-53 (TcdA, TcdB), and 017 (TcdB) (Table S2, Table

2). Non-toxigenic isolates belonged to ribotypes 009, 035 and 439 (Table S2, Table 2). The two most prevalent ribotypes (n=5 animals) were 017 (N=8 isolates) and 009 (N=7).

The two isolates of animal #29 belonged to different ribotypes (439 and 005). For all other pairs of isolates originating from the same animal, both shared the same ribotype (Table 2), suggesting phylogenetic proximity and raising the possibility of a single clonal lineage colonizing the animal gut.

Antimicrobial susceptibility

All strains were susceptible to metronidazole and vancomycin (Table 2). The median Minimum Inhibitory Concentration (MIC) was 0.13 mg/L for metronidazole and 0.5 mg/L for vancomycin. The strains least susceptible to metronidazole (MIC=0.38 mg/L) or vancomycin (MIC=1.0 mg/L) were of ribotype 017 and 126 respectively. The second lowest level of vancomycin susceptibility (MIC=0.75 mg/L) was shared by one third of strains, mostly of ribotypes 017 and 009 (Table 2). The pairs of ribotype 017 strains originating from three animals (#32, #36 and #37) were poorly susceptible to each antibiotic (Table 2).

***C. difficile* putative circulation**

We cross-analyzed *C. difficile* ribotype and the location of the corresponding Equidae (Table S1, Figure 1, Table 2). In each of two premises between Caen and Pont-Audemer, few animals shared the same ribotype: I) two co-located Equidae necropsied in 2019 (#42 and #43) displayed toxigenic strains of ribotype 020, and ii) four co-located Equidae, necropsied in 2019 (#5, #18 and #21) and 2020 (#61), all carried strains of ribotype 009. The same clonal lineage could therefore have contaminated several animals of each premises, which could represent a cluster of transmission.

CDI or carriage

We tested the presence of free toxins in animal samples using a standard enzymatic immuno-assay against TcdA and TcdB. In order to check its specificity when applied to equine digestive contents rather than human stools, we included samples expected to be toxin-negative: i) all samples displaying a non-toxigenic *C. difficile* (Table 3) and ii) twenty-seven *C. difficile*-negative samples, including all those from animals having died from endo-enterotoxaemia and fifteen CDL (Table3, Table S3). No toxins could be detected in any of them, while *C. difficile* glutamate dehydrogenase (GDH) was positive in most of the former, as expected (Table S3, Table 3).

The samples of all Equidae displaying a toxigenic *C. difficile* were assayed. CDI was demonstrated in four animals, which displayed signs of diarrhea (CDL in the colon) and a toxigenic ribotype 017 (#32, #36 and #37) or 126 (#3), as free toxins, together with GDH, were detected in their samples (Table 3, Figure 2). The remaining 16 Equidae were asymptomatic carriers (Table 3), as indicated by their clinical picture in most cases. i) Eight of them had no signs of diarrhea. ii) The signs of diarrhea of seven animals could have been caused by another entero-pathogen in five

cases or by another intestinal disease that caused animal death (acute folded colon and parasite infection) (Table S1). Finally, only in the last case, the absence of detectable toxins was necessary to demonstrate asymptomatic carriage.

In the equine subpopulation displaying a toxigenic *C. difficile*, animals with a CDI and asymptomatic carriers were similar according to sex, age, and non-pathognomonic signs of endo-enterotoxaemia or diarrhea, but they significantly differed according to breed and antibiotic treatment (Table 1). In contrast to a minority of asymptomatic carriers, all or most animals with a CDI respectively were Trotters or had received antibiotics (Table S1, Table 1). This confirmed antibiotics to be a risk factor for CDI in the equine population.

Cause of animal death

We examined the cause of death of the four animals with CDI (Table S1). The fulminant death of animal #3, a 15-days-old foal, was independent from CDI and due to the intussusception of small intestine (Table S1). Animal #37 died from multiple, respiratory, musculoskeletal and digestive infections including endo-enterotoxaemia, possibly due to *Klebsiella pneumoniae*, identified in lungs, lung abscess and hock muscle, and *C. difficile* (Table S1).

CDI caused the endo-enterotoxaemia and death of two animals #32 and #36 (Table S1), and such a prevalence was consistent with previous data. The same ribotype, ribotype 017, was implicated in both cases. It had also probably caused the death of one preliminary studied animal, which had been necropsied in 2018, displayed *post-mortem* observed signs of diarrhea (CDL) and endo-enterotoxaemia and had previously received antibiotics (Table S1, Table 2).

Discussion

C. difficile prevalence in necropsied Equidae

A *C. difficile* prevalence in French necropsied Equidae of 27% was unanticipated by most professionals in Normandie. And yet, it was similar to the prevalence in European live horses, which ranged from 4-33% for all animals to 5-63% for ill animals [10]. The dead condition of animals apparently did not result in any major bias here, probably because animal death causes were very diverse (Table S1). Moreover, 20% of the population displayed at least one *C. difficile* toxigenic strain, a preoccupying finding with respect to dissemination. Most of these animals (16%) were asymptomatic carriers that would have remained unnoticed without our study.

C. difficile diversity

C. difficile strains isolated from Equidae belonged to diverse, common or at least already described ribotypes [10, 30]. Almost all toxigenic ribotypes found in Equidae were common in humans. Ribotypes 020, 078, 126 and 005 on the one hand and 017 and 020 on the other hand were among the Top10 and Top20 ribotypes found in patients in Europe in the 2013-2014 period [30]. French Equidae could therefore contribute to the dissemination of clinically important ribotypes. And yet, the epidemic ribotype 027, responsible for outbreaks in North America and Europe in the 2000's [1], and described in horses [19], was not identified here. This might be correlated to the changing *C. difficile* epidemiology and the decrease of 027 prevalence in humans since the 2010's [31], notably in France (https://www.pasteur.fr/fr/file/44921/download) [30, 32].

Most ribotypes evidenced here have also previously been detected in many *C. difficile* animal hosts [10, 11]. Ribotypes 126, 020 and 012 had been found in farm animals [10]. Of note, ribotype 078, prevalent in animals, notably pigs [10], was rather uncommon here (N=1 isolate). However, the phylogenetically close ribotype 126 [33, 34], with the same characteristic *tcdC* deletion (Table 2) was also represented (N=3). In Bretagne, a French region close to Normandie, porcine, bovine and poultry manure from biogas plants almost exclusively provided ribotypes 078 and 126 [26]. Moreover, the non-toxigenic ribotype 009 has previously been described in Czech horses [35] and companion animals [36].

Overall, ribotypes found in Equidae can widely be found in environmental sources. As many as nine of them 005, 009, 012, 017, 020, 078, 126, 439 and AI-53 have been detected in *C. difficile* invertebrate vectors, like mollusks [37, 38]. Ribotypes 020, 012, 078 and 126 have been found in potatoes [39, 40] and vegetables in Europe [11], ribotype 035 in soils in Iraq [41] and ribotype 009 in sandboxes in Spain [42]. Finally, according to the noteworthy diversity of ribotypes identified here, Equidae suggested that could be important players in *C. difficile* environmental dissemination and transmission to other hosts [8, 43].

Circulation and putative transmission of *C. difficile* ribotypes

The co-localization of animals sharing one ribotype suggested transmission clusters. In the stud farm contaminated by ribotype 009, spores should have persisted for months as the last animal

(#61) was born after the death of the others (#5, #18, #21) (Table S1). In the other premises, two animals (#42 and #43) were asymptomatic carriers of ribotype 020 (Table 3). The CDI cases caused by ribotype 017 (#32, #36 and #37) were from different stud farms (Figure 1). However, they occurred in a small area (Figure 1) during a 6-weeks period (Table S1) and could represent a local infection cluster. The full understanding of *C. difficile* transmission and phylogeny would require further genome-wide investigations.

Presence of several entero-pathogens

C. difficile could be present in Equidae with intestinal disorders or diseases, as more *C. difficile*-positive animals than negative ones showed signs of diarrhea or of endo-enterotoxaemia (Table S1, Table 1). Indeed, in four of the seven asymptomatic carriers of a toxigenic *C. difficile* another clostridial pathogen could be identified: *C. perfringens* alone in animal #43 and together with *P. sordellii* in animals #29, #42 and #53 (Table S1). Co-colonization or co-infection by both *C. difficile* and *C. perfringens* had previously been described in foals [44], horses [45], dogs [46], piglets [47] and even humans [48].

Co-colonization by a toxigenic and a non-toxigenic *C. difficile*

In our study, the most complicated case was animal #29, which displayed three pathogens and two *C. difficile* strains (Table S1), one of ribotype 005 in the intestine and a non-toxigenic one of ribotype 439 in the caecum (Table 2). Such a co-colonization has already been described. Strains of ribotype 009 could be found together with toxigenic strains in companion animals [36]. Moreover, non-toxigenic strains prevented or reduced clinical signs of CDI in animal models [49-52]. A non-toxigenic strain of ribotype 009 not only prevented CDI in experimentally challenged piglets [53] but also reduced the occurrence of CDI in piglets of a commercial farm [47].

Equidae with a CDI and risk factors

To reach a high standard diagnosis of CDI in necropsied Equidae, we combined toxin detection, a method of high specificity and positive prediction value, to the amplification of toxin genes, a method of high sensitivity and negative prediction value, as recommended in European hospitals [4, 54]. Toxin detection is rather rare in animals, even though it had sometimes been performed in Equidae [55-57], even after *post-mortem* examination [58]. Here, toxin detection definitely demonstrated CDI in 4% of the whole equine population.

All Equidae with a CDI in contrast to a minority of asymptomatic carriers of a toxigenic *C. difficile* were Trotters (Table 1, Table S1). The condition of their microbiota could have favored their CDI. Indeed, most Trotters with CDI had previously been treated by antibiotics, which could have promoted microbiota dysbiosis (Table 1, Table S1). On the contrary, none of the six Thoroughbred carriers of a toxinogenic strain had received antibiotics and the four Thoroughbred having received antibiotics were carriers of a non-toxinogenic strain (Table 1, Table S1) [47].

Virulence of ribotypes 126 and 017

CDIs in Equidae had been caused by isolates of ribotypes 126 or 017. Of note, the former were the least susceptible to vancomycin, and the latter the least susceptible to metronidazole and the second least susceptible to vancomycin. This suggested that Equidae treatments against CDIs could select strains more difficult to eradicate and able to better persist after treatment.

The ribotypes found here to be virulent in Equidae differ by their reported prevalence. Ribotype 126 is common in humans in Europe, it was the sixth most prevalent ribotype in patients in 2013-2014, with a prevalence of 5.1% [30], and it is also close to ribotype 078, which is well-known as prevalent in animals. Ribotype 017, although well-known as an emerging ribotype in Asia [59], is less frequent in Europe, where it was the 12th most prevalent ribotype in 2013-2014, with a prevalence of 1.9%. In France, its prevalence was <1% in 2017 [30] and 2020 (https://www.pasteur.fr/fr/sante-publique/CNR/les-cnr/bacteries-anaerobies-botulisme/rapports-d-activite), and it was undetected in ten hospitals in 2022 [32]. As ribotype 017 has also been described as relatively uncommon in animals [59], its prevalence in French Equidae (5%) was noticeable. Such a prevalence could fall within the range of normal fluctuations, or alternatively, ribotype 017 could have caused a local outbreak or even could be emerging in Equidae and/or in Normandie. In any case, its virulence was of concern and French Equidae, as a reservoir for *C. difficile* clinical ribotypes, would deserve careful monitoring in a One Health perspective.

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500

Figure Legends

Figure 1. Geographical location of *C. difficile*-positive Equidae in Normandie and PCR-ribotypes of their strains

C. difficile-positive Equidae are shown in a map of the French region Normandie according to the latitude and longitude coordinates of their premises (Table S1). *C. difficile* ribotype (Table 2) is indicated in a box, and the number of animals bearing this ribotype in each premises (Table S1) is shown between brackets. The pathogenicity of *C. difficile* strains is indicated by a colour code: non-toxinogenic strains in green, and toxigenic strains in either orange or red depending on whether they were asymptomatically carried or responsible for a CDI. The location of animals is marked by symbols depending on the toxin profiles of their corresponding strains: squares for a three-toxin profile (TcdA TcdB and CDT, for strains of ribotypes 078 and 126), circles for two-toxin profile (TcdA and TcdB, for strains of ribotypes: 005, 012, 020, 181 and AI-53), triangles for a TcdB profile (for strains of ribotype 017) and finally diamonds for non-toxigenic strains (ribotypes: 009, 035 and 439) (Table 2).

Figure 2. Diagnosis of *C. difficile*-positive Equidae according to their strain characteristics

C. difficile-positive animals necropsied from 2019 to 2021 are shown as a wedge of a colour pie chart. *C. difficile* strains, *C. difficile*-positive animals and diagnosis are indicated in four concentric rings.

First (core): Toxin profile of *C. difficile* strains (see Table 2). Non-toxigenic strains ('Non-Tox') and toxigenic strains ('Tox') are indicated on a green or orange background, respectively. 'Both' is indicated when a non-toxigenic strain and a toxigenic strain were co-colonizing the same animal (#29, bi-coloured wedge). The toxin profile of toxigenic strains is specified using the following abbreviations: A for toxin A (TcdA), B for toxin B (TcdB), CDT for binary toxin and '?' when the capacity of the strain to encode CDT (the presence of *cdt* genes) has not been tested.

Second: Strain ribotypes (according to WEBRIBO database) (Table 2)

Third: Animal identification number

Fourth (outer ring): Diagnosis (Table 3). CDI, asymptomatic carriage of a toxigenic strain or non-toxigenic strain carriage are on a red, orange or green background, respectively.

Table legends

Table 1. Equine population: data summary

The equine population composition is shown. The number of necropsied animals is indicated according to year or main animal data: i) individual data (sex, age, breed), ii) *post-mortem* observations (signs of diarrhea, endo-enterotoxaemia), iii) *ante-mortem* data (previous antibiotic treatment or hospitalization) (Table S1). The equine population composition is statistically analyzed according to: i) *C. difficile* presence or not, ii) strain pathogenicity or not for *C. difficile*-positive animals and finally iii) the diagnosis of CDI or asymptomatic carriage, for animals positive for a toxigenic strain.

Table 2. *C. difficile* strains isolated from French necropsied Equidae

All preserved strains are listed and their origin (animal and sample) indicated. All data about molecular characterization: multiplex PCR results, toxin profile, PCR-ribotype and Multi Locus Sequence Type (MLST) are shown. Strain susceptibility to vancomycin or metronidazole (MICs) is also indicated.

Table 3. Diagnosis of CDI or carriage

All the data of *C. difficile*-positive Equidae leading to a diagnosis with respect to *C. difficile* infection or not are indicated: i) signs of putative diarrhea as inferred from *post-mortem* observed watery consistency of intestinal samples, ii) presence of a toxigenic strain and iii) free toxin detection. Animals are grouped according to diagnosis: *C. difficile* infection, asymptomatic carriage of a toxigenic strain or carriage of a non-toxigenic strain.

All preserved strains are listed with the corresponding animal and sample indicated in each case.

Supporting information

Table S1. Registered data on all Equidae necropsied from 2019 to 2021 in Normandie

Table S2. MLST characterization of the first isolated strains

Statements

Data availability statement

All data are available in the manuscript.

Funding statement

This work was funded by IFCE (Institut Français du Cheval et de l'Equitation, France, https://www.ifce.fr, Research project 2019-009-Rech CloDifEqui), ANSES and INRAE.

Conflict of interest statement

The authors declare that they have no conflict of interest.

Ethics statement

The study was performed in compliance with institutional guidelines for research on necropsied animals and was approved by the scientific committee of IFCE (Institut Français du Cheval et de l'Equitation, France, https://www.ifce.fr) that funded the project. Data about animals and owners are anonymous.

Author contributions

SP and IP designed the whole project, submitted it to IFCE for funding. JT, NF and MBR performed animal necropsies and intestinal content sampling, registered and analyzed *post-mortem* data. JT established the cause of animal death. KM performed bacteriological and molecular analyses to identify pathogens. SP, KM, FD, SK, LB and IP searched and isolated *C. difficile* strains and performed the primary molecular characterization of strains. FB performed final molecular characterization and free toxin detection. SP and IP analyzed all data. IP wrote the manuscript and SP, JT, FB, FD, SK and IP edited it. All authors read the final version of the manuscript and agreed to submit it.

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Figure 1

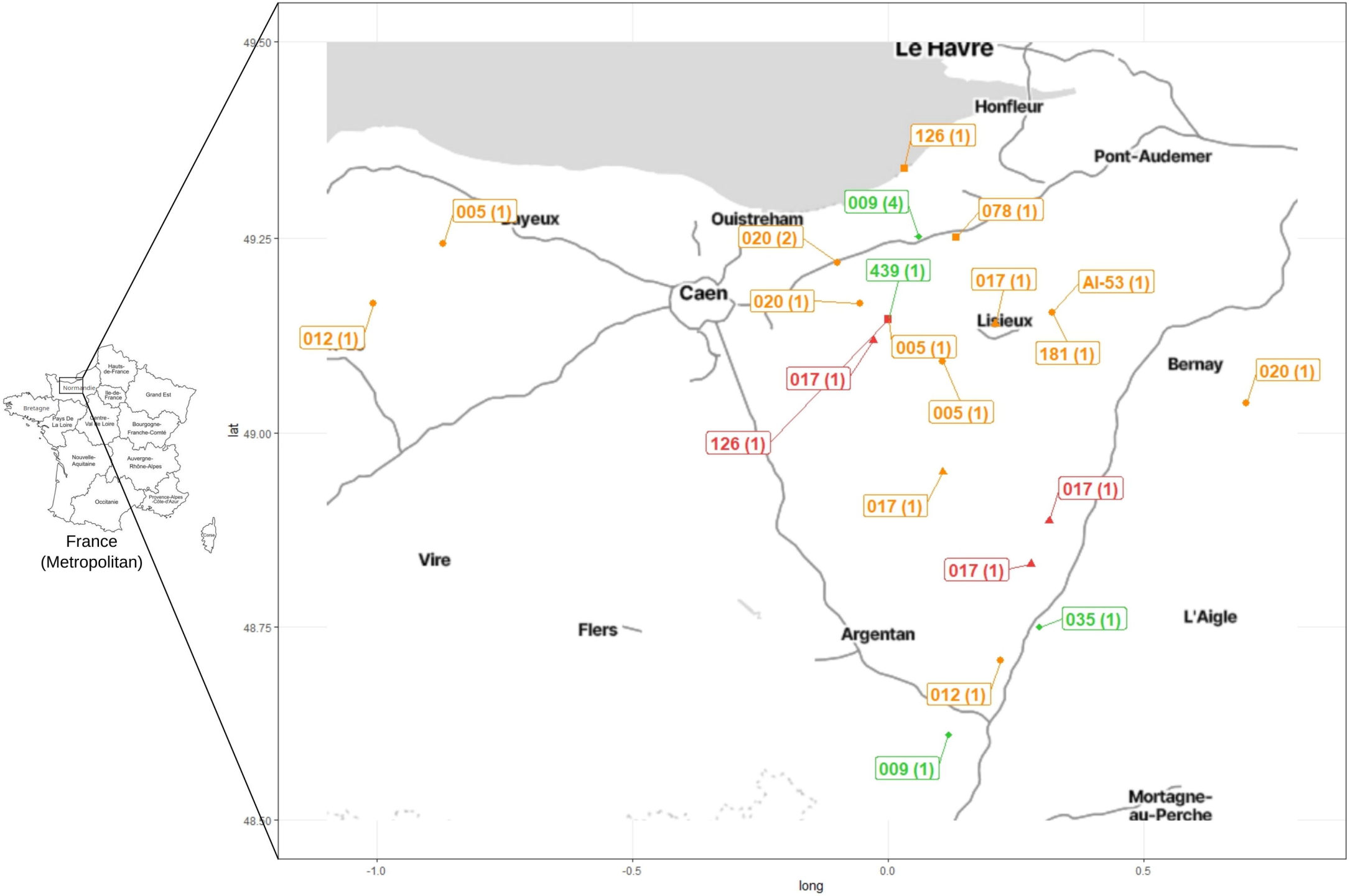
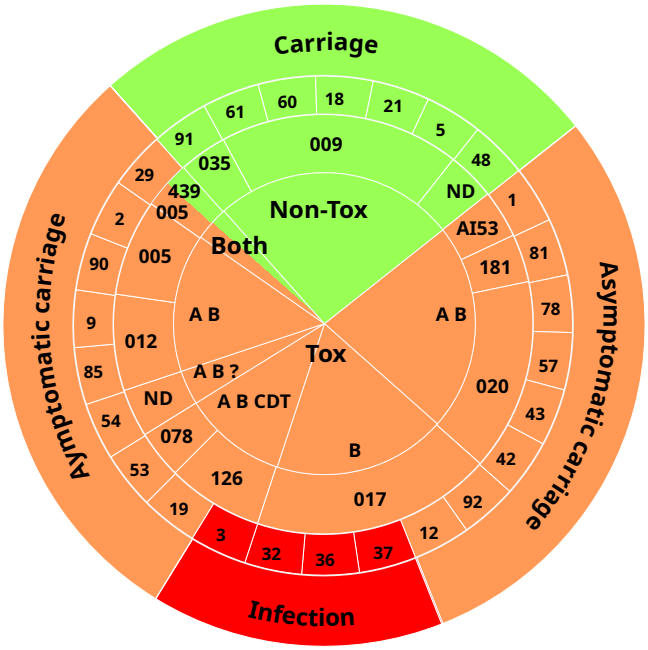

Le Havre
Honfleur
Pont-Audemer
Ouistreham
Caen
Lisieux
Bernay
Vire
Flers
Argentan
L'Aigle
Mortagne-au-Perche
126 (1)
005 (1)
009 (4)
078 (1)
020 (2)
439 (1)
017 (1)
AI-53 (1)
020 (1)
012 (1)
005 (1)
181 (1)
020 (1)
017 (1)
005 (1)
126 (1)
017 (1)
017 (1)
017 (1)
035 (1)
012 (1)
009 (1)
49.50
49.25
49.00
48.75
48.50
lat
-1.0
-0.5
0.0
0.5
long
Normandie
Hauts-de-France
Ile-de-France
Grand Est
Bretagne
Pays De La Loire
Centre-Val de Loire
Bourgogne-Franche-Comté
Nouvelle-Aquitaine
Auvergne-Rhône-Alpes
Occitanie
Provence-Alpes-Côte-d'Azur
Corse
France
(Metropolitan)

Figure 2



Carriage
Asymptomatic carriage
Infection
Asymptomatic carriage
Non-Tox
Both
Tox
009
18
21
5
48
ND
1
AI53
81
181
78
A B
57
020
43
42
92
12
B
017
37
36
32
3
126
19
53
078
54
ND
85
A B CDT
A B ?
9
012
90
005
2
005
29
439
91
035
61
60

Table 1. Main data on necropsied Equidae

	Presence of *C. difficile*			*C. difficile* strain				Diagnosis (Toxigenic *C. difficile*)		
	No	Yes	P-value	Non-toxigenic	Toxigenic	Both	P-value	Carriage	Infection	P-value
Year										
2019	32	15	0.60	3	11	1	0.91	8	4	0.30
2020	28	8		3	5	0		5	0	
2021	13	4		1	3	0		3	0	
Animal data										
Sex										
Female	46	21	0.44	7	13	1	0.34	11	3	1.00
Male, gelding	26	6		0	6	0		5	1	
Unknown	1	0								
Age										
Foal	33	15	0.55	6	9	0	0.42	6	3	0.79
Yearling	10	1		0	1	0		1	0	
Young	5	2		0	2	0		2	0	
Adult	25	9		1	7	1		7	1	
Race										
Thoroughbred	30	12	0.87	6	6	0	0.15	6	0	**0.04**
Trotter	23	8		1	6	1		3	4	
Saddlebred horse	9	2		0	2	0		2	0	
Other (6 breeds)	9	5		0	5	0		5	0	
Unknown	2	0								
• Thoroughbred versus Other	30 / 43	12 / 15	0.82	6 / 1	6 / 13	0 / 1	**0.02**	6 / 10	0 / 4	0.27
• Trotter versus Other	23 / 50	8 / 19	1.0	1 / 6	6 / 13	1 / 0	0.29	3 / 13	4 / 0	**0.007**
***Post-mortem* signs of**										
Diarrhea										
One sample per animal: CAE	60	11	**0.0001**	3	8	0	1.0	8	0	0.12
Two samples per animal: CAE + CDL	13	16		4	11	1		8	4	
Endo/enterotox□mia										
Yes	7	13	**0.0001**	3	9	1	1.0	7	3	0.58
No	65	14		4	10	0		9	1	
Risk factors										
Previous antibiotic treatment										
Yes	24	11	0.8332	5	6	0	0.3124	3	3	**0.0144**
No	43	14		2	11	1		12	0	
Unknown	6	2		0	2	0		1	1	
Previous hospitalization										
Yes	22	11	0.5731	4	7	0	0.6483	7	0	0.2487
No	49	16		3	12	1		9	4	
Unknown	2	0		3	12	1		9	4	

Table 2: Molecular characterization of *C. difficile* strains

Strain Name	Isolated from		Pathogenicity							Type		Antibio-resistance	
			Genotype (established by PCR)						Toxin profile [f]				
	Equidae n°	Intestinal sample	*tpi* [a]	*tcdA* [b]	*tcdB* [a]	lok [c]	*tcdC* [d] deletion	*cdt* [e]		Ribotype [g] (WEBRIBO)	MLST [h]	MIC Vancomycin (mg/L) [i]	MIC Metronidazol (mg/L) [i]
***Cd* E-3**	3	CAE	+	+	+	-	-39	+	TcdA TcdB CDT	**126**	ST11	**1.00**	0.09
***Cd* E-3-CDL**		CDL										**1.00**	0.06
***Cd* E-19**	19	CAE										0.38	0.05
***Cd* E-53**	53	CAE[§]	+	+	+	-	-39	+	TcdA TcdB CDT	**078**	nd	0.50	0.09
***Cd* E-2**	2	CAE	+	+	+	-	0	T	TcdA TcdB	**005**	nd	**0.75**	0.13
***Cd* E-90**	90	CAE									nd	0.25	0.05
***Cd* E-29-CDL**	29*	CDL									nd	**0.75**	0.13
***Cd* E-9**	9	CAE	+	+	+	-	0	T	TcdA TcdB	**012**	ST54	0.50	0.05
***Cd* E-85**	85	CAE									nd	0.50	0.05
***Cd* E-42**	42	CAE	+	+	+	-	0	T	TcdA TcdB	**020**	nd	**0.75**	0.19
***Cd* E-42-CDL**		CDL									nd	0.50	0.13
***Cd* E-43**	43	CAE									ST2	0.50	0.19
***Cd* E-43-CDL**		CDL									ST2	0.50	**0.25**
***Cd* E-57**	57	CAE[§]									nd	0.50	0.13
***Cd* E-78**	78	CAE[§]									nd	0.50	0.19
***Cd* E-81-CDL**	81	CDL[§]	+	+	+	-	0	T	TcdA TcdB	**181**	nd	0.38	0.19
***Cd* E-1**	1	CAE	+	+	+	-	0	T	TcdA TcdB	**AI-53**	nd	0.25	0.13
***Cd* E-12**	12	CAE	+	del	+	-	0	-	TcdB	**017**	ST37	0.50	0.09
***Cd* E-32**	32	CAE									ST37	**0.75**	**0.38**
***Cd* E-32-CDL**		CDL									ST37	**0.75**	0.19
***Cd* E-36**	36	CAE									ST37	**0.75**	**0.25**
***Cd* E-36-CDL**		CDL									ST37	**0.75**	0.19
***Cd* E-37**	37	CAE									nd	**0.75**	**0.25**
***Cd* E-37-CDL**		CDL									ST37	**0.75**	**0.38**
***Cd* E-92**	92	CAE									nd	0.50	**0.25**
CdE-0-CDL	0[¤]	CDL									nd	0.38	0.032
***Cd* E-5**	5	CAE	+	-	-	+	-	-	Non toxigenic	**009**	ST3	0.50	0.09
***Cd* E-18**	18	CAE									ST3	**0.75**	0.13
***Cd* E-18-CDL**		CDL									ST3	0.50	0.09
***Cd* E-21**	21	CAE									nd	0.13	0.09
***Cd* E-60**	60	CAE									nd	**0.75**	0.09
***Cd* E-60-CDL**		CDL									nd	0.50	0.06
***Cd* E-61-CDL**	61	CDL[§]									nd	**0.75**	0.06
***Cd* E-91**	91	CAE	+	-	-	+	-	-	Non toxigenic	**035**	nd	0.13	0.13
***Cd* E-29**	29*	CAE	+	-	-	+	-	-	Non toxigenic	**439**	nd	0.50	0.05

[a] *tpi, tcdB* PCR-fragment of wild-type size (+) or no amplification (-)

[b] *tcdA* PCR-fragment of wild-type size (+) or of shorter size indicative of a deletion (del) or no amplification (-)

[c] lok PCR-fragment of 117 bp (+, i. e. PaLoc deletion, non toxigenic strain) or no amplification (-)

[d] *tcdC* deletion of 39 bp (-39) or no deletion (0; i. e. PCR-fragment of wild-type size) or no amplification (-)

[e] *cdt* PCR-fragment of wild-type size (+) or truncated PCR-fragment indicative of a pseudogene (T) or no amplification (-)

[f] Toxin profile: the combination of toxines that the strain is able to produce, according to its PCR-established genotype

[g] Ribotype: PCR ribotype assigned according to the WEBRIBO database (WRT)

[h] MLST: Multi-Locus Sequence Type; nd: not determined

[i] MIC: Minimum Inhibitory concentration; the two highest MIC values are in bold

* animal co-colonized by two different strains (one recovered from CAE and the other from CDL sample)

[¤] animal necropsied at the end of 2018 (preliminary experiment)

[§] the single animal sample that provided a *C. difficile* strain (out of the two recovered ones)

Table 3: Diagnosis of *C.difficile* infection or carriage

Necropsied Equ n°	Intestinal sample	*C. difficile* Strain [a]	Pathogenicity [b]	Toxin profile [c]	Ribotype (WRT)[b, d]	Enzymatic Immuno Assay GDH [d]	Free Toxins [d] (TcdA TcdB)	Diagnosis [d]
3	CAE	*Cd* E-3	**Toxigenic**	TcdA TcdB CDT	126	+	+	***C. difficile* infection**
	CDL	*Cd* E-3-CDL	**Toxigenic**	TcdA TcdB CDT	126	+	+	
32	CAE	*Cd* E-32	**Toxigenic**	TcdB	017	+	+ (very weak)	
	CDL	*Cd* E-32-CDL	**Toxigenic**	TcdB	017	+	+ (very weak)	
36	CAE	*Cd* E-36	**Toxigenic**	TcdB	017	+	+	
	CDL	*Cd* E-36-CDL	**Toxigenic**	TcdB	017	+	+	
37	CAE	*Cd* E-37	**Toxigenic**	TcdB	017	+	+	
	CDL	*Cd* E-37-CDL	**Toxigenic**	TcdB	017	+	+	
19	CAE	*Cd* E-19	**Toxigenic**	TcdA TcdB CDT	126	-	-	Asymptomatic carriage
53	CAE	*Cd* E-53	**Toxigenic**	TcdA TcdB CDT	078	-	-	
	CDL	none	NA		NA	-	-	
54	CAE	none	NA		NA	-	-	
	CDL	not preserved	**Toxigenic**	TcdA TcdB ?	ND	-	-	
2	CAE	*Cd* E-2	**Toxigenic**	TcdA TcdB	005	+ (very weak)	-	
29*	CAE	*Cd* E-29	Non Toxigenic	None	439	+ (very weak)	-	
	CDL	*Cd* E-29-CDL	**Toxigenic**	TcdA TcdB	005	+ (very weak)	-	
90	CAE	*Cd* E-90	**Toxigenic**	TcdA TcdB	005	-	-	
9	CAE	*Cd* E-9	**Toxigenic**	TcdA TcdB	012	+	-	
85	CAE	*Cd* E-85	**Toxigenic**	TcdA TcdB	012	+	-	
42	CAE	*Cd* E-42	**Toxigenic**	TcdA TcdB	020	-	-	
	CDL	*Cd* E-42-CDL	**Toxigenic**	TcdA TcdB	020	-	-	
43	CAE	*Cd* E-43	**Toxigenic**	TcdA TcdB	020	+	-	
	CDL	*Cd* E-43-CDL	**Toxigenic**	TcdA TcdB	020	+	-	
57	CAE	*Cd* E-57	**Toxigenic**	TcdA TcdB	020	-	-	
	CDL	none	NA		NA	-	-	
78	CAE	*Cd* E-78	**Toxigenic**	TcdA TcdB	020	-	-	
	CDL	none	NA		NA	-	-	
81	CAE	none	NA		NA	-	-	
	CDL	*Cd* E-81-CDL	**Toxigenic**	TcdA TcdB	181	-	-	
1	CAE	*Cd* E-1	**Toxigenic**	TcdA TcdB	AI-53	-	-	
12	CAE	*Cd* E-12	**Toxigenic**	TcdB	017	-	-	
92	CAE	*Cd* E-92	**Toxigenic**	TcdB	017	-	- +	
5	CAE	*Cd* E-5	Non toxigenic		009	+	-	Carriage
18	CAE	*Cd* E-18	Non toxigenic		009	+	-	
	CDL	*Cd* E-18-CDL	Non toxigenic		009	+	-	
21	CAE	*Cd* E-21	Non toxigenic		009	-	-	
60	CAE	*Cd* E-60	Non toxigenic		009	+	-	
	CDL	*Cd* E-60-CDL	Non toxigenic		009	+	-	
61	CAE	none	NA		NA	-	-	
	CDL	*Cd* E-61-CDL	Non toxigenic		009	+	-	
91	CAE	*Cd* E-91	Non toxigenic		035	+	-	
48	CAE	none	NA		NA	-	-	
	CDL	not preserved	Non toxigenic		ND	-	-	

* co-colonized animal

¤ animal necropsied in 2018 (preliminary set-up experiment), no free toxin detection assay was performed

[a] Strain name (preserved strain) or not preserved (detected but not preserved strain) or none (no detectable *C. difficile*)

[b] NA: not applicable

[c] Toxin profile: toxins that the strain is able to produce according to PCR results

? is indicated when strain ability to produce CDT remains unknown (no PCR targeting *cdt* genes was performed)

[d] ND: not determined