Clostridioides difficile in Equidae necropsied in northwestern France, between 2019 and 2021

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Abstract

Clostridioides difficile is an anaerobic, spore-forming entero-pathogen, causing diarrhea in humans and animals. We evaluated *C. difficile* occurrence, diversity, circulation and virulence in necropsied Equidae (n=100) in northwestern France (Normandy), from 2019 to 2021. We systematically recovered all cecal contents and any watery (possibly diarrheal) intestinal contents. We isolated *C. difficile* strains, determined their toxin gene profile by PCR and established their PCR-ribotype according to the WEBRIBO database. We also performed free toxin detection.

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 common ribotypes: i) 078 and 126 (able to produce toxin A, toxin B and binary toxin), ii) 005, 012, 020, 181 and AI-53 (toxins A and B), and iii) 017 (toxin B). Non-toxigenic isolates were of ribotypes 009, 035 and 439. Ribotypes 017 and 009 were predominant (n=5). In two cases, Equidae of the same premises shared the same ribotype, either 020 or 009. Free toxins were detected in four animals: they displayed signs of diarrhea and a *C. difficile* of ribotype 126 (n=1) or 017 (n=3) as the only detected entero-pathogen, suggesting a *C. difficile* infection (CDI). Three of them had received antibiotics. Two had died from an entero-toxic infection, for which *C. difficile* ribotype 017 was the only identified cause.

French Equidae were found to display common pathogenic *C. difficile*, and ribotype 017 was highly virulent. These findings are of concern from a One Health perspective.

Importance

C. difficile, a major entero-pathogen widely disseminated in the environment, is a One Health issue. Animals are raising concern as human contamination sources. Equidae are in close contact with humans and also develop *post*-antibiotic and healthcare-associated CDIs. The systematic survey of Equidae necropsied from 2019 to 2021 in the first breeding region in France, revealed that 20% harbored pathogenic strains. These belonged to clinically important ribotypes, raising the possibility of cross-species, possibly zoonotic transmission. Free toxins, whose testing is rare in animals, were detected in four animals with diarrhea signs and a toxigenic *C. difficile* as the only identified entero-pathogen, suggesting CDI. In two of them, *C. difficile* ribotype 017 was the only identified cause of entero-toxic disease and death.

French Equidae could play a role in the dissemination of pathogenic *C. difficile* and notably ribotype 017. They should be surveilled carefully from a One Health perspective.

Keywords

Clostridioides difficile, One Health, necropsied Equidae, prevalence, diversity, toxin detection

Introduction

Clostridioides difficile is a spore-forming anaerobe responsible for diarrhea and colitis (1). *C. difficile,* whose spores are transmitted by the fecal-oral route, is a major cause of healthcare-associated diarrhea and a rising cause of community-acquired infections. *C. difficile* infections (CDIs) can be severe, with complications and recurrences, and life threatening. Toxin A (TcdA) and Toxin B (TcdB) are responsible for symptoms, with TcdB alone being sufficient to cause disease. The binary toxin (CDT) has an accessory role (2). Non-toxigenic strains are unable to cause disease, while toxigenic strains can cause disease or be asymptomatically carried (3). Antibiotics, hospitalization and age increase the risk of developing CDI (1). Antibiotics notably induce microbiota dysbiosis, which favors spore germination, vegetative multiplication, and ultimately, toxin production. Healthcare-associated CDIs are treated with fidaxomicin and vancomycin (4), which can contribute to microbiota dysbiosis and favor *C. difficile* re-emergence, possibly from persisting biofilms (5, 6). In this context, the last resort treatment is fecal microbiota transplant (FMT) (4).

C. difficile is a One Health issue (7-10). It causes diarrhea in a wide range of wild, farm and companion animals (8, 9, 11-13). *C. difficile* strains can be transmitted between host species (10, 14-16). Equidae, which are monogastric mammals, are closely interacting with humans and relevant to zoonotic and anthropo-zoonotic transmission. Importantly, in these animals of high economic value (https://www.ifce.fr) like in humans, antibiotics treatments and hospitalization increase the risk of developing CDI. In Equidae*, C. difficile* is also responsible for outbreaks and sporadic cases (17-21). Both Equidae and humans are treated with vancomycin and have long been treated with metronidazole, which is still used in animals but replaced with fidaxomicin for hospitalized patients. Like humans, Equidae can also be treated with FMT (22). The main difference between Equidae and humans as *C. difficile*-infected populations is age. While <3-years-old children are mainly asymptomatic carriers (3), CDI is common in foals (17, 18).

Data about *C. difficile* importance in horses (7, 9, 11, 19, 23) remain scarce in France. *C. difficile* testing is not systematic, and the detection of a toxigenic strain is not sufficient to diagnose CDI (3). Here, we studied *C. difficile* in Equidae necropsied in Normandy, the first breeding region in France (12,600 foals and 145,000 Equidae in 2021, https://www.ifce.fr). Necropsy (24) provided the opportunity both to recover digestive contents from a population with diverse clinical pictures, and to get access to detailed animal data including death cause. Our objective was to evaluate *C. difficile* occurrence, diversity, putative circulation and virulence in French Equidae.

Materials and methods

Equine necropsy

ANSES (PhEED) performs equine necropsies at the request of breeders or veterinarians to determine the cause of animal death. The standardized procedure includes examination of the cadaver, evisceration and organ examination. Animal identification data, previous treatments, including antibiotics in the two-three weeks before death, necropsy observations, pathogen identification and death cause are registered (https://sitesv2.anses.fr/fr/minisite/resumeq/presentation-de-resumeq) (24).

In 2018, we chose five necropsied Equidae with *post-mortem* signs of endo-enterotoxaemia and diarrhea and collected their watery intestinal contents for a pilot study. Then, from May 2019 to August 2021, we studied all one hundred necropsied animals (except fetus and stillborn foals), whatever clinical picture or death cause. This total number, after two years characterized by Covid-19 and lockdowns, was relatively low, matching the previous annual average. Animal digestive contents were systematically recovered in the caecum (CAE; n=100), and in case of a watery consistency, in any other intestine segment (CDL; n=30), notably the colon. Aliquots of all digestive contents except a cecal one (129 samples in 2019-2021) were preserved at 4°C, -20°C and -70°C.

This study followed institutional guidelines and was approved by the scientific committee of IFCE (https://www.ifce.fr) that funded the project. All data are anonymous.

Standard pathogen testing

In case of *post-mortem* observed signs of infection, the veterinarian requested routine pathogen testing in relevant samples (Table S1). For intestinal infections, usual testing in digestive contents was as follows. *Escherichia coli* colonies, after detection on Eosin Methylene Blue plates, were streaked on blood plates to see hemolysis. *Salmonella* spp. were isolated on XLD plates (Thermo Scientific). *C. difficile* was detected on ChromID (BioMérieux) under anaerobiosis, and *Clostridium perfringens* and *Paeniclostridium sordellii* on Columbia agar containing 5% sheep blood (BioMérieux). Matrix-Assisted Laser Desorption/Ionization Time-Of-Flight (MALDI-TOF) Mass Spectrometry (MS) (MBT Smart mass spectrometer, Compass and FlexControl softwares, Bruker Daltonics) confirmed identification. Rotavirus was identified by PCR. Intestinal parasite communities were identified and quantified by visual inspection (25).

Specific *C. difficile* testing

Here, we also systematically performed *C. difficile* testing. A sample of any digestive content was streaked onto ChromID or CLO plates (BioMérieux) and incubated for 48h at 37°C under anaerobiosis. In case of a negative result, a 1g-aliquot was inoculated in BHI broth supplemented by cysteine (0.1%), yeast extract (5 g/L), taurocholate (1 g/L), D-cycloserine (250 µg/mL) and cefoxitin (8 µg/mL) (OXOID), and incubated for 3-7 days (enrichment) before plating onto ChromID. Suspicious, irregular and/or black colonies were screened by PCRs targeting *tpi*, *tcdA* and *tcdB* genes (26, 27). Identification was confirmed by MALDI-TOF MS. For each confirmed strain, a single

colony per positive sample was grown in BHI broth at 37°C under anaerobiosis to be preserved in glycerol (17%) at -70°C, except in two cases: two strains (non-toxigenic in animal #48 and toxigenic in #54) characterized without being preserved, could not be recovered later.

C. difficile characterization

All preserved isolates (N=34 from n=25 Equidae in 2019-2021 and N=1 in 2018) were characterized by multiplex PCR targeting: i) *tpi* gene, ii) wild-type or truncated toxin genes and iii) *lok* fragment characterizing non-toxigenic strains, followed by capillary gel electrophoresis (28). For ribotyping, PCR profiles after capillary gel-based electrophoresis (29) were analyzed with GeneMapper software (Thermo Fischer Scientific, Villebon-sur-Yvette, France) and ribotypes assigned according to database WEBRIBO (https://webribo.ages.at/). The first isolates were characterized by Multi Locus Sequence Typing (MLST) (30).

Susceptibility to Vancomycin and to metronidazole

The Minimum Inhibitory Concentration (MIC) was determined 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 24 h at 37°C.

Toxin detection

C. difficile toxin A, toxin B and glutamate dehydrogenase (GDH) were detected in digestive contents using an Enzymatic Immuno-Assay (Quick Check Complete, Alere), according to manufacturer's instructions.

Statistical analysis

We used Fisher's exact test to analyse the equine population composition, after excluding unknown values, and Mann-Whitney test to compared strain MICs.

Results

Necropsied Equidae

We studied all Equidae (except fetuses and stillborn foals) (n=100) necropsied at ANSES in Normandy from May 2019 to August 2021, without any selection (Table S1). All animals except three originated from Normandy. Most were females (67%) and <1-year-old animals (59%), with foals (<6-months) representing half of the population (48%) (Table 1). Nine breeds were present, mostly Thoroughbred (42%), Trotters (31%) and Saddlebred horses (11%) (Table 1). Animals had died from very diverse (>30) causes including infectious or non-infectious diseases and trauma (Table S1). The first and second most prevalent causes of death were respectively endo-enterotoxaemia (12%), an enteric disease caused by toxin-producing bacteria, like Salmonella and Clostridia (20), and infection by *Rhodococcus equi* (9%), common in foals (31).

C. difficile prevalence

After recovering all cecal contents and any watery intestinal contents, possibly indicating diarrhea, all preserved contents (99 CAE and 30 CDL) were tested for *C. difficile*, directly or after enrichment. Candidate colonies were screened by standard PCRs (26, 27) and confirmed by MALDI-TOF. Twenty-seven 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).

Pathogenicity

Thirty-four *C. difficile* isolates were preserved from twenty-five Equidae and named '*Cd*E' (Table 2). They form the first French library of *C. difficile* strains originating from Equidae, CloDifEqui (Table 2). Their pathogenicity profile was characterized by multiplex PCR, which revealed non-toxigenic isolates (N=9) and toxigenic ones (N=25) with three profiles (*tcdA*$^+$ *tcdB*$^+$ *cdt*$^+$, *tcdA*$^+$ *tcdB*$^+$ and *tcdB*$^+$) (Table 2). The former isolates originated from seven animals, including six Thoroughbred, and the latter from nineteen animals of six breeds (Table S1). One animal (#29) was co-colonized by a non-toxigenic strain (CAE) and a toxigenic one (CDL). In the eight other animals with two isolates, both were either non-toxigenic (#18, #60) or toxigenic with the same profile (#3, #32, #36, #37, #42, #43) (Table 2).

Ribotype diversity

CloDifEqui isolates were assigned to eleven ribotypes and four clades (1, 2, 4 and 5) (Table 2, Table S2) (29). Toxigenic isolates were of eight ribotypes: 126 and 078 (*tcdA*$^+$ *tcdB*$^+$ *cdt*$^+$ and same *tcdC* truncation), 020, 005, 012, 181 and AI-53 (*tcdA*$^+$ *tcdB*$^+$), and 017 (*tcdB*$^+$). Non-toxigenic isolates belonged to ribotypes 009, 035 and 439. The two most prevalent ribotypes (n=5 animals) were 017 (N=8 isolates) and 009 (N=7).

Both toxigenic and non-toxigenic strains of animal #29 respectively belonged to ribotype 005 and 439. In all other pairs of isolates originating from the same animal, both were of the same ribotype (Table 2).

Susceptibility to metronidazole and to vancomycin

All strains were susceptible to the antibiotics used to treat CDI in Equidae, metronidazole and vancomycin (MICs lower than EUCAST breakpoints: 2 mg/L in both cases; https://www.eucast.org/fileadmin/src/media/PDFs/EUCAST_files/Breakpoint_tables/v_15.0_Breakpoint_Tables.pdf) (Table 2). The median MIC was 0.13 mg/L for metronidazole and 0.5 mg/L for vancomycin. Strains of ribotype 017 and 126 showed the highest MIC of metronidazole (0.38 mg/L) and of vancomycin (1.0 mg/L), respectively. Strains of ribotype 017 significantly displayed higher MIC of metronidazole (median=0.25) than other strains (median=0.09; p=0.007). This suggested that metronidazole treatment against equine CDIs could select strains more difficult to eradicate and able to better persist.

Co-localization of Equidae sharing the same ribotype

We cross-analyzed Equidae localization and *C. difficile* ribotypes (Figure 1, Table S1). In two premises between Caen and Pont-Audemer, some animals shared the same ribotype: i) 020, for two co-localized Equidae necropsied in 2019 (#42 and #43), and ii) 009 for four co-localized Equidae necropsied in 2019 (#5, #18 and #21) and 2020 (#61). This suggested that a single clonal lineage could have contaminated each premises environment. Each premises could represent a transmission cluster, with a common source contaminating animals or animals contaminating each other. The full understanding of *C. difficile* transmission and phylogeny would require further genome-wide investigations.

Toxin production

We tested the presence of free toxins in equine digestive contents using a standard enzymatic immuno-assay. To check its specificity when applied to these contents rather than human stools, we included samples expected to be toxin-negative: i) all ten samples displaying a non-toxigenic *C. difficile* (Table 3) and ii) twenty-seven *C. difficile*-negative samples (fifteen CDL and all samples from animals with endo-enterotoxaemia signs) (Table 3, Table S1, Table S3). No toxins could be detected in any of them, while GDH was positive in seven of the former (Table 3, Table S3).

Free toxins and GDH were detected in the two samples of four animals (#3, #32, #36 and #37). They displayed diarrhea signs (CDL in the colon) and a toxigenic *C. difficile* (ribotype 126 or 017), as the only identified entero-pathogen (Table 3, Figure 2). This highly suggested *C. difficile* virulence and animal CDI. The sixteen Equidae with a toxigenic *C. difficile* but no toxins (Table 3) were carriers. Half of them showed no diarrhea signs (#1, #2, #9, #12, #19, #85, #90 and #92), suggesting asymptomatic carriage. For almost all others (except #57), diarrhea signs might be due to another intestinal pathogen (*C. perfringens* in #43, *C. perfringens* or *P. sordellii* in #29 and #42,

C. perfringens, *P. sordellii* or rotavirus in #53, a parasite in #78) or disease (torsion of the large colon in #54 and intestinal lymphoma in #81) (Table S1).

In Equidae displaying a toxigenic *C. difficile*, the subpopulations displaying *C. difficile* toxins or not 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). Three out four toxin-positive animals (all Trotters) compared to three out of sixteen carriers of a toxigenic *C. difficile* had received antibiotics (Table S1, Table 1). Antibiotics treatments could therefore have increased the risk of developing CDI.

Cause of animal death

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

For the last two animals, #32 and #36, death was due to endo-enterotoxaemia (Table S1). *C. difficile*, which had produced its toxins (Table 3), was the only identified and most probable cause of death (Table S1). The resulting prevalence of *C. difficile* as the cause of animal death (2%) was of the same range as the previously measured ones. Of note, the same ribotype 017 was implicated in both cases and probably also in one case in 2018: animal #0, which had died from endo-enterotoxaemia after an antibiotic treatment, displayed signs of diarrhea and *C. difficile* as the only entero-pathogen (Table 2, Table S1).

Discussion

C. difficile prevalence and diversity

Here, we evaluated the presence of *C. difficile* in Equidae necropsied in France for the first time. *C. difficile* prevalence in these Equidae having died from diverse, mainly non-intestinal diseases (Table S1) was of 27%. This level was similar to, and in some cases lower than, the reported prevalence in European live horses: 4-33% for healthy animals and 5-63% for sick animals with intestinal illnesses (9).

C. difficile strains from Equidae belonged to eleven already described ribotypes (9, 32). The historical epidemic ribotype 027 (1), described in horses (19), was not identified here, probably because of the changing *C. difficile* epidemiology and 027 prevalence decrease (33)(32, 34). Most ribotypes of CloDifEqui toxigenic strains are common in humans: ribotypes 020, 078, 126 and 005 were in the Top10 ribotypes in European patients in 2013-2014, and ribotypes 017 and 012 in the Top20 (32). In Czech horses, toxigenic strains were of five ribotypes, including only one in the Top20 (32). French Equidae could therefore contribute to the dissemination of diverse, clinically important and prevalent ribotypes.

Most ribotypes of CloDifEqui toxigenic strains are also common in animals (9, 11). Ribotypes 126, 020 and 012 had been found in farm animals (9). Ribotype 078, known as prevalent in animals, notably pigs (9), was relatively uncommon here (N=1 isolate), but the phylogenetically close ribotype 126 was represented (N=3) (Table 2) (35). Of note, the ribotypes of most CloDifEqui toxigenic strains have been detected in diverse sources in Europe, including molluscs (005, 012, 017, 020, 078, 126 and AI-53) (36), potatoes and vegetables (012, 020, 078 and 126) (11, 37). The ubiquity and prevalence of ribotypes identified here suggested that Equidae could contribute to the dissemination, circulation and transmission of pathogenic *C. difficile*, a One Health issue.

Toxin production

After PCR amplification of toxin genes, a method of high sensitivity and negative prediction value, we performed toxin detection, a method of high specificity and positive prediction value, as recommended for CDI diagnosis in European patients (3), but rarely performed in animals including Equidae (38-41). Four animals with both diarrhea signs and a toxigenic *C. difficile* were positive for toxins, suggesting virulence *in vivo* and CDI. *C. difficile* carriers represented 23% of the population (Table 3, Figure 2), a high and previously overlooked level.

In Equidae, *C. difficile* presence (Table 1, Table S1), and even carriage (p-value <0.005), were significantly correlated to animal signs of diarrhea or endo-enterotoxaemia. These signs in *C. difficile* carriers were independent from *C. difficile* toxins and nevertheless more frequent than in negative animals (12/23 versus 15/75). *C. difficile* carriers also more frequently harbored another entero-pathogen (bacterium, virus, parasite) than negative animals (7/23 versus 9/73) (Table S1), and these pathogens might be responsible for diarrhea signs. The most prevalent was *C. perfringens* in four *C. difficile* carriers (Table S1). Host co-colonization or co-infection by *C. difficile* and *C. perfringens* have been described in horses (42, 43), dogs (44), pigs (45) and humans (46).

In this study, the most complex case was animal #29, which displayed three clostridial entero-pathogens and two *C. difficile* strains, one toxigenic and the other non-toxigenic (Table S1, Table 2). Animal co-colonization by a toxigenic strain and a non-toxigenic one, notably of ribotype 009, has been reported in companion animals (47). Non-toxigenic strains can prevent CDI or reduce its severity in animal models (48) and a ribotype 009 strain can protect model and commercial piglets (45). Whether CloDifEqui non-toxinogenic strains, especially of ribotype 009, could provide protection against a toxigenic strain would deserve further investigations.

C. difficile had produced toxins in four Trotters and at least three had received antibiotics (Table 1, Table S1). Antibiotic treatments could have favored microbiota dysbiosis, *C. difficile* outgrowth and virulence. Of note, among the eight *C. difficile* carriers having received antibiotics, five displayed non-toxinogenic strains unable to cause disease.

C. difficile strains that had produced toxins in Equidae were of ribotypes 126 and 017. Ribotype 126 was the sixth most prevalent ribotype in European patients in 2013-2014 (32). It is also, together with its close relative 078, prevalent in production animals, notably pigs (9, 49), and in biogas plants using animal manure in another French region, Brittany (28). Ribotype 017, which is prevalent in Asia (50), is common in European patients (32) but infrequent and sometimes undetected in French ones (32)(34). As ribotype 017 is also uncommon in animals (9, 50) and was not detected in biogas plants in Brittany (28), its prevalence in French Equidae (5%), and especially those with a toxigenic *C. difficile* (25%) was noticeable. Moreover, its virulence in three close animals (Figure 1) within a short period (Table S1) raised the possibility of a local infection cluster. Finally, French Equidae, able to disseminate clinically important and prevalent ribotypes of *C. difficile*, would deserve careful monitoring from a One Health perspective.

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485

Figure Legends

Figure 1. Geographical localization of *C. difficile*-positive Equidae

Each *C. difficile*-positive Equidae is indicated on a map of Normandy, according to the latitude and longitude coordinates of its stud farm (Table S1). The ribotype of the corresponding *C. difficile* (Table 2) is indicated in a box, and when several animals harbored this ribotype in the same premises, their number appears between brackets (Table S1). *C. difficile* pathogenicity and virulence are shown using the following color code: green for non-toxinogenic, orange for toxigenic and carried, and finally red for toxigenic and virulent (having produced toxins). Different symbols refer to *C. difficile* toxin gene profiles: squares for *tcdA*$^+$ *tcdB*$^+$ *cdt*$^+$ (ribotypes 078 and 126), circles for *tcdA*$^+$ *tcdB*$^+$ (ribotypes 005, 012, 020, 181 and AI-53), triangles for *tcdB*$^+$ (ribotype 017) and diamonds for non-toxigenic strains (ribotypes 009, 035 and 439) (Table 2).

Figure 2. *C. difficile* virulence or carriage in *C. difficile*-positive Equidae

All *C. difficile*-positive animals are shown as a wedge of a pie chart. For each positive animal, the pathogenicity, ribotype and virulence of *C. difficile* are indicated in four concentric rings.

First (core): *C. difficile* pathogenicity (Table 2). Non-toxigenic ('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 gene profile of toxigenic strains is specified using the following abbreviations: A for *tcdA*$^+$, B for *tcdB*$^+$, C for *cdt*$^+$ and '?' when the presence of *cdt* genes has not been tested.

Second: *C. difficile* ribotype (according to WEBRIBO database) (Table 2).

Third: Animal identification number.

Fourth (outer ring): Virulence (Toxin production, Table 3), carriage of a toxigenic strain and carriage of a non-toxigenic strain are on a red, orange and green background, respectively.

Table 1. Equine population: data summary

C. difficile	**Presence**			**Pathogenicity**				**Toxin production**		
Equidae	No	Yes	P-value	Non-toxigenic	Toxigenic	Both	P-value	No	Yes	P-value
Necropsy 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	0.23	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	0.80	6	6	0	0.15	6	0	**0.038**
Trotter	23	8		1	6	1		3	4	
Saddlebred horse	9	2		0	2	0		2	0	
Other breeds	9	5		0	5	0		5	0	
*Unknown**	*2*	*0*								
Thoroughbred / all other breeds	30 / 41	12 / 15	1.0	6 / 1	6 / 13	0 / 1	**0.026**	6 / 10	0 / 4	0.27
Trotter / all other breeds	23 / 48	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	59	11	**0.0002**	3	8	0	1.0	8	0	0.12
Two samples per animal: CAE + CDL	14	16		4	11	1		8	4	
Endo/enterotox□mia										
Yes	7	13	**0.00008**	3	9	1	1.0	7	3	0.58
No	65	14		4	10	0		9	1	
*Unknown**	*1*	*0*								
Risk factors										
Previous antibiotic treatment										
Yes	24	11	0.48	5	6	0	0.18	3	3	**0.025**
No	43	14		2	11	1		12	0	
*Unknown**	*6*	*2*		*0*	*2*	*0*		*1*	*1*	
Previous hospitalization										
Yes	22	11	0.47	4	7	0	0.65	7	0	0.25
No	49	16		3	12	1		9	4	
*Unknown**	*2*	*0*		*3*	*12*	*1*		*9*	*4*	

Table 1 Legend

This contingency table shows the composition of the equine population (number of necropsied animals). In lines, Equidae are categorized according to: i) necropsy year, ii) identity data (sex, age, breed), iii) *post-mortem* observations (signs of diarrhea or of endo-enterotoxaemia), iv) *ante-mortem* data (previous antibiotic treatment or hospitalization) (Table S1). In columns, Equidae are categorized according to *C. difficile*: i) its presence or not, assessed in the whole equine population (n=100), ii) *C. difficile* pathogenicity or not, in the subpopulation of *C. difficile*-positive animals (n=27) and finally iii) its production of toxins (virulence) or not, in the subpopulation of animals with a toxigenic *C. difficile* (n=20).

* the information was missing in the necropsy register (Table S1). In these cases, the number of animals is indicated in italics for full and complete information, but these animals were excluded from the statistical analysis using Fisher's exact test. Statistical significance: P-values < 0.05, which are shown in bold, on a grey background

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

C. difficile strain Name	Equidae Number	Digestive content	Multiplex PCR *tpi* [a]	*tcdA* [b]	*tcdB* [a]	lok [c]	*tcdC* [d] deletion	*cdt* [e]	Profile [f]	Ribotype [g] (WEBRIBO)	MLST [h]	Vancomycin MIC (mg/L) [i]	Metronidazole MIC (mg/L) [i]
2019-2021													
*Cd*E-3	3	CAE	+	+	+	-	-39	+	***tcdA*$^+$ *tcdB*$^+$ *cdt*$^+$**	126		**1.00**	0.09
*Cd*E-3-CDL	3	CDL	+	+	+	-	-39	+		126	ST11	**1.00**	0.06
*Cd*E-19	19	CAE	+	+	+	-	-39	+		126		0.38	0.05
*Cd*E-53	53	CAE[§]	+	+	+	-	-39	+		078	nd	0.50	0.09
*Cd*E-1	1	CAE	+	+	+	-	0	T		AI-53	nd	0.25	0.13
*Cd*E-81-CDL	81	CDL[§]	+	+	+	-	0	T		181	nd	0.38	0.19
*Cd*E-42	42	CAE	+	+	+	-	0	T		020	nd	**0.75**	0.19
*Cd*E-42-CDL	42	CDL	+	+	+	-	0	T		020	nd	0.50	0.13
*Cd*E-43	43	CAE	+	+	+	-	0	T		020	ST2	0.50	0.19
*Cd*E-43-CDL	43	CDL	+	+	+	-	0	T		020	ST2	0.50	**0.25**
*Cd*E-57	57	CAE[§]	+	+	+	-	0	T		020	nd	0.50	0.13
*Cd*E-78	78	CAE[§]	+	+	+	-	0	T		020	nd	0.50	0.19
*Cd*E-9	9	CAE	+	+	+	-	0	T		012	ST54	0.50	0.05
*Cd*E-85	85	CAE	+	+	+	-	0	T		012	nd	0.50	0.05
*Cd*E-2	2	CAE	+	+	+	-	0	T	***tcdA*$^+$ *tcdB*$^+$**	005	nd	**0.75**	0.13
*Cd*E-29-CDL*	29	CDL	+	+	+	-	0	T		005	nd	**0.75**	0.13
*Cd*E-90	90	CAE	+	+	+	-	0	T		005	nd	0.25	0.05
*Cd*E-12	12	CAE	+	del	+	-	0	-		017	ST37	0.50	0.09
*Cd*E-32	32	CAE	+	del	+	-	0	-		017	ST37	**0.75**	**0.38**
*Cd*E-32-CDL	32	CDL	+	del	+	-	0	-		017	ST37	**0.75**	0.19
*Cd*E-36	36	CAE	+	del	+	-	0	-		017	ST37	**0.75**	**0.25**
*Cd*E-36-CDL	36	CDL	+	del	+	-	0	-		017	ST37	**0.75**	0.19
*Cd*E-37	37	CAE	+	del	+	-	0	-		017	nd	**0.75**	**0.25**
*Cd*E-37-CDL	37	CDL	+	del	+	-	0	-		017	ST37	**0.75**	**0.38**
*Cd*E-92	92	CAE	+	del	+	-	0	-		017	nd	0.50	**0.25**
*Cd*E-29*	29	CAE	+	-	-	+	-	-		439	nd	0.50	0.05
*Cd*E-91	91	CAE	+	-	-	+	-	-		035	nd	0.13	0.13
*Cd*E-5	5	CAE	+	-	-	+	-	-	**Non toxigenic**	009	ST3	0.50	0.09
*Cd*E-18	18	CAE	+	-	-	+	-	-		009	ST3	**0.75**	0.13
*Cd*E-18-CDL	18	CDL	+	-	-	+	-	-		009	ST3	0.50	0.09
*Cd*E-21	21	CAE	+	-	-	+	-	-		009	nd	0.13	0.09
*Cd*E-60	60	CAE	+	-	-	+	-	-		009	nd	**0.75**	0.09
*Cd*E-60-CDL	60	CDL	+	-	-	+	-	-		009	nd	0.50	0.06
*Cd*E-61-CDL	61	CDL[§]	+	-	-	+	-	-		009	nd	**0.75**	0.06
2018													
CdE-0-CDL	0	CDL	+	del	+	-	0	-	***tcdB*$^+$**	017	nd	0.38	0.032

Table 2 Legend.

All preserved strains are listed. Their origin (animal and digestive content) is indicated and whether they have been isolated after enrichment is specified. For each strain, all results of molecular characterization: multiplex PCR results, PCR-ribotype and Multi Locus Sequence Type (MLST, Table S3), and the Minimal Inhibitory Concentration (MIC) of vancomycin and metronidazole are shown.

[a] PCR-fragment of wild-type size ('+') or absence of amplification ('-')

[b] PCR-fragments of wild-type size ('+') or of shorter size indicative of a deletion ('del') or absence of amplification ('-')

[c] PCR-fragment of 117 bp ('+', i. e. Pathogenicity Locus deletion and non-toxigenic strain) or no amplification ('-')

[d] 39bp-deletion ('-39') or absence of deletion ('0', i. e. PCR-fragment of wild-type size) or absence of amplification ('-')

[e] PCR-fragment of wild-type size ('+') or truncated PCR-fragment ('T', i. e. presence of a pseudogene) or absence of amplification ('-')

[f] toxin gene profile according to multiplex PCR results: toxin genes of wild-type size or non-toxigenic

[g] PCR-ribotype assigned according to the WEBRIBO database

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

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

[*] the two strains co-colonizing the same animal

[§] the single digestive content (among the two ones recovered from the animal) from which *C. difficile* was isolated

Table 3. Free toxin detection

Equidae n°	Digestive content	Strain [a]	Enrichment [b]	Pathogenicity [c]	Ribotype	GDH [d]	Free toxins [d] Toxins A and B
3	CAE	*Cd*E-3	-	**Toxigenic**	126	+	**+**
	CDL	*Cd*E-3-CDL	-	**Toxigenic**	126	+	**+**
32	CAE	*Cd*E-32	-	**Toxigenic**	017	+	**+** (very weak)
	CDL	*Cd*E-32-CDL	-	**Toxigenic**	017	+	**+** (very weak)
36	CAE	*Cd*E-36	-	**Toxigenic**	017	+	**+**
	CDL	*Cd*E-36-CDL	-	**Toxigenic**	017	+	**+**
37	CAE	*Cd*E-37	-	**Toxigenic**	017	+	**+**
	CDL	*Cd*E-37-CDL	-	**Toxigenic**	017	+	**+**
29*	CAE	*Cd*E-29	+	Non Toxigenic	439	+ (very weak)	-
	CDL	*Cd*E-29-CDL	-	**Toxigenic**	005	+ (very weak)	-
42	CAE	*Cd*E-42	+	**Toxigenic**	020	-	-
	CDL	*Cd*E-42-CDL	+	**Toxigenic**	020	-	-
43	CAE	*Cd*E-43	-	**Toxigenic**	020	+	-
	CDL	*Cd*E-43-CDL	-	**Toxigenic**	020	+	-
53	CAE	*Cd*E-53	+	**Toxigenic**	078	-	-
	CDL	none	NA	NA	NA	-	-
54	CAE	none	NA	NA	NA	-	-
	CDL	not preserved	-	**Toxigenic**	ND	-	-
57	CAE	*Cd*E-57	-	**Toxigenic**	020	-	-
	CDL	none	NA	NA	NA	-	-
78	CAE	*Cd*E-78	+	**Toxigenic**	020	-	-
	CDL	none	NA	NA	NA	-	-
81	CAE	none	NA	NA	NA	-	-
	CDL	*Cd*E-81-CDL	+	**Toxigenic**	181	-	-
1	CAE	*Cd*E-1	+	**Toxigenic**	AI-53	-	-
2	CAE	*Cd*E-2	+	**Toxigenic**	005	+ (very weak)	-
9	CAE	*Cd*E-9	-	**Toxigenic**	012	+	-
12	CAE	*Cd*E-12	-	**Toxigenic**	017	-	-
19	CAE	*Cd*E-19	-	**Toxigenic**	126	-	-
85	CAE	*Cd*E-85	-	**Toxigenic**	012	+	-
90	CAE	*Cd*E-90	+	**Toxigenic**	005	-	-
92	CAE	*Cd*E-92	-	**Toxigenic**	017	-	-/+ (very weak)
18	CAE	*Cd*E-18	-	Non toxigenic	009	+	-
	CDL	*Cd*E-18-CDL	-	Non toxigenic	009	+	-
48	CAE	none	NA	NA	NA	-	-
	CDL	not preserved	-	Non toxigenic	ND	-	-
60	CAE	*Cd*E-60	-	Non toxigenic	009	+	-
	CDL	*Cd*E-60-CDL	-	Non toxigenic	009	+	-
61	CAE	none	NA	NA	NA	-	-
	CDL	*Cd*E-61-CDL	+	Non toxigenic	009	+	-
5	CAE	*Cd*E-5	-	Non toxigenic	009	+	-
21	CAE	*Cd*E-21	-	Non toxigenic	009	-	-
91	CAE	*Cd*E-91	-	Non toxigenic	035	+	-

Table 3 Legend.

The results of free toxin detection are shown for the digestive contents of all *C. difficile*-positive Equidae. For each Equidae, identification number and the nature of recovered digestive content(s) (CAE and possibly CDL, in bold) are indicated. For each digestive content, *C. difficile* strain and pathogenicity are shown together with the results of toxin and GDH detection (enzymatic immuno-assay). Animals are listed according to *C. difficile* pathogenicity and to their diarrhea signs (watery CDL content).

[a] Strain name or 'not preserved' (an initially detected strain that could not be recovered later) or 'none' (absence of *C. difficile* detection)

[b] The method used to isolate the strain: Enrichment ('+') or not ('-', i. e. direct inoculation on plates)

[c] Toxigenic (in bold) or non-toxigenic strain

[d] detection ('+') or very weak detection ('+ very weak' for GDH, '-/+ very weak' for toxins) or absence of detection ('-')

NA: not applicable (not isolate)

ND: not determined

* co-colonized animal

Supporting information

Table S1. Animal data

Table S2. Sequence Type (MLST) of CloDifEqui strains

Table S3. Toxin detection in *C. difficile*-negative animals

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Authorship contribution statement

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 manuscript and agreed to submit it.

Declaration of competing interest

The authors declare that they have no conflict of interest.

Data availability

All data are available in the manuscript.

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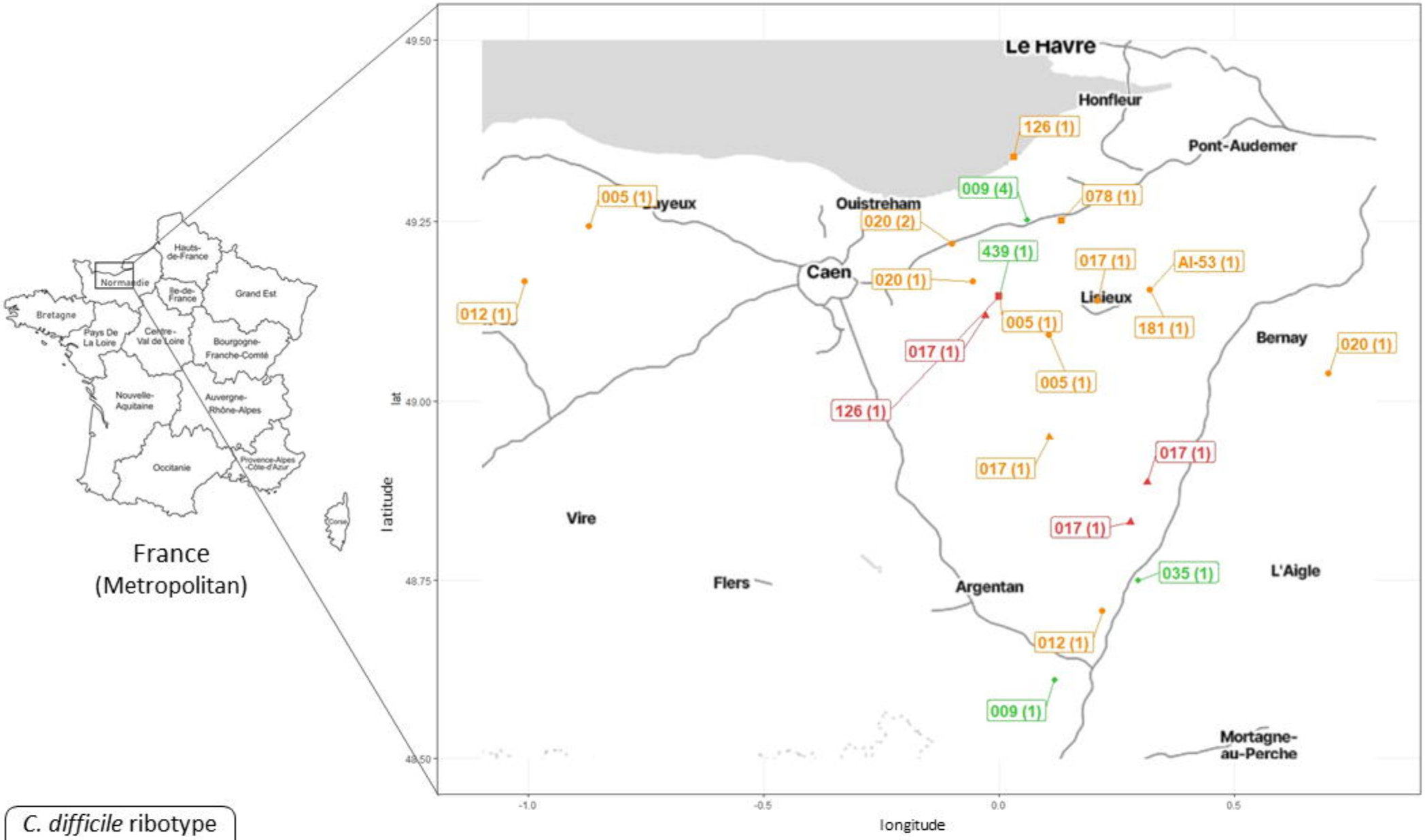
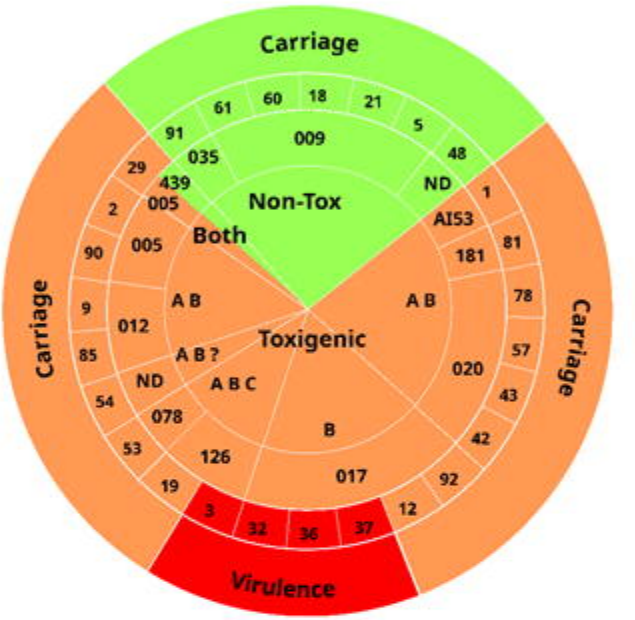


C. difficile ribotype
(number of animals)

Green = non toxigenic
Orange = toxigenic/carried
Red = toxigenic/virulent
(Toxins⁺)

◇ = lok
Δ = *tcdB*⁺
○ = *tcdA*⁺ *tcdB*⁺
□ = *tcdA*⁺ *tcdB*⁺ *cdt*⁺

Figure 2



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