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

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Abstract

Clostridioides difficile is an anaerobic, spore-forming entero-pathogen, causing diarrhea in humans and animals. We studied French Equidae as a model host to evaluate *C. difficile* occurrence, diversity, virulence and circulation. We took advantage of necropsies of Equidae in northwestern France (Normandy), 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.

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: 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. Ribotypes 017 and 009 were predominant (n=5). In two premises, some Equidae shared the same ribotype, 020 or 009, suggesting potential transmission clusters. In four animals with signs of diarrhea and toxigenic isolates, the detection of free toxin demonstrated *C. difficile* infection. These isolates were of ribotype 126 (n=1) or 017 (n=3) and the least susceptible to vancomycin. Two animals died from their infection by ribotype 017, which is emerging in Asia but infrequent in Europe.

French Equidae represented an underestimated reservoir of pathogenic *C. difficile* ribotypes and one of them was highly virulent, both preoccupying findings in 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 exposed to the same risk factors. The systematic survey of Equidae necropsied from 2019 to 2021 in the first breeding region in France, revealed that 20% harboured pathogenic strains. These belonged to prevalent, clinically important ribotypes, raising the possibility of zoonotic transmission. A high-standard diagnosis of *C. difficile* infection (CDI), recommended in European hospitals, but rare in animals, revealed CDI in 4% of the population, and prior antibiotic treatment was a major risk factor. A ribotype known as emerging in Asia was highly virulent and caused a local infection cluster.

French Equidae as underestimated reservoirs of *C. difficile* and notably of ribotype 017 are of concern in a One Health perspective and should be carefully surveilled.

Keywords

Clostridioides difficile, One Health, Equidae, prevalence, diversity, transmission, diagnosis, infection/carriage

Introduction

C. difficile is a spore-forming anaerobe responsible for diarrhea and colitis (1). It is a major cause of health-care associated diarrhea and 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. TcdA and TcdB toxins are responsible for symptoms, even though TcdB alone is sufficient for disease, and CDT, the binary toxin, 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 are the main risk factors for CDI (1). Antibiotics notably induce microbiota dysbiosis, which favors spore germination and vegetative multiplication. CDIs are treated by fidaxomicin and vancomycin (4), which contribute to dysbiosis and therefore relapses. Spores and biofilms could contribute to persistence between relapses (5, 6). The last resort treatment is fecal microbiota transplant (FMT) (4).

C. difficile is a One Health issue (7-10). It causes diarrhea in most, wild, farm and companion animals (8, 9, 11-13). *C. difficile* strains can jump between hosts, leading to inter-species transmission (10, 14-16). Equidae, which are closely interacting with humans, are relevant to zoonotic and anthropo-zoonotic transmission. Importantly, the healthcare of these monogastric animals of high economic value (https://www.ifce.fr) is careful, and they have the same main risk factors for CDI as humans, antibiotics and hospitalization. In both 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). As infected populations, their main difference is age. While <3-years-old children are mainly asymptomatic carriers (3), CDI can affect foals (17, 18).

Data about *C. difficile* importance in horses (7, 9, 11, 19) 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 (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, together with the cause of their 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 (ANSES, PhEED) are performed to determine the cause of animal death, using a standardized procedure (23). It includes examination of the cadaver, evisceration and organ examination. Animal identification data, previous treatments, necropsy observations, pathogen identification and death cause are registered (https://sitesv2.anses.fr/fr/minisite/resumeq/pr%C3%A9sentation-de-resumeq) (23).

In 2018, the intestinal contents of five necropsied Equidae with *post-mortem* signs of endo-enterotoxaemia and diarrhea were collected for a pilot study. Then, for all animals necropsied from May 2019 to August 2021 (except fetus and stillborn foals), digestive contents were systematically recovered in the caecum, and in case of a watery consistency, in any other intestine segment, notably the colon. All samples except one caecal content (129) were preserved as aliquots 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 search for entero-pathogens

Escherichia coli colonies were isolated on Eosin Methylene Blue plates and streaked on blood plates to see hemolysis, and *Salmonella* spp. on XLD plates (Thermo Scientific). *C. difficile* was detected on ChromID (BioMérieux) and *Clostridium perfringens* and *Paeniclostridium sordellii* on Columbia agar containing 5% sheep blood (BioMérieux), under anaerobiosis. 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.

Systematic *C. difficile* search

Any digestive sample was directly streaked onto 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 (0.1%), yeast extract (5 g/L), taurocholate (1 g/L), D-cycloserine (250 µg/mL) and cefoxitin (8 µg/mL) (OXOID), allowed enrichment before ChromID plating. Suspicious, irregular and/or black colonies were screened by PCRs targeting *tpi*, *tcdA* and *tcdB* genes (24, 25). Identification was 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 but not immediately preserved strains (non-toxigenic in animal #48 and toxigenic in #54) could not be recovered again.

C. difficile characterization

All preserved isolates (N=34 from n=25 Equidae) 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 (26). For ribotyping, PCR profiles after capillary gel-based electrophoresis (27) 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 (28).

Vancomycin or metronidazole susceptibility

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 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 to diagnose CDI, as recommended in Europe (3).

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 all one hundred Equidae (except fetuses and stillborn foals) necropsied at ANSES in Normandy from 2019 to 2021, whatever clinical picture or death cause (Table S1). All animals except three originated from Normandy (Table S1). Most were females (67%) and <1-year-old animals (59%), 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 were collected from i) the caecum (CAE) in all cases (n=100 animals) and ii) any other intestinal segment in case of watery consistency (CDL), possibly indicating diarrhea (n=30) (Table S1). All preserved digestive contents were tested for *C. difficile* presence, directly or after enrichment. Candidate colonies were screened by standard PCRs (24, 25) 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

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 (26). Non-toxigenic isolates (N=9) originated from seven animals, including six Thoroughbred, while toxigenic isolates (N=25), with one- to three-toxin profiles, originated from nineteen animals of six breeds (Table 2, Table S1). One animal (#29) was co-colonized by a non-toxigenic strain (CAE) and a toxigenic one (CDL). In all other animals with two isolates, both were either non-toxigenic or toxigenic with the same toxin profile (Table 2).

Ribotype diversity

CloDifEqui isolates were assigned to eleven ribotypes and four clades (1, 2, 4 and 5) (Table 2, Table S2) (27). Toxigenic isolates were of eight ribotypes: 126 and 078 (three-toxin profile: 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 (Table 2, Table S2). 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 ribotypes 439 and 005. For all other pairs originating from the same animal, both isolates shared the same ribotype (Table 2), suggesting their phylogenetic proximity and raising the possibility of a single clonal lineage colonizing animal gut.

Metronidazole or vancomycin susceptibility

All strains were susceptible to metronidazole and vancomycin (Table 2). The median 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. Most ribotype 017 strains were poorly susceptible to each antibiotic (Table 2).

Putative circulation

We cross-analyzed *C. difficile* ribotype and the corresponding Equidae location (Table S1, Figure 1, Table 2). In two premises between Caen and Pont-Audemer, few animals shared the same ribotype: i) two co-located Equidae necropsied within a few days in 2019 (#42 and #43) displayed toxigenic ribotype 020, and ii) four co-located Equidae, necropsied in 2019 (#5, #18 and #21) and 2020 (#61), carried ribotype 009. This suggests that a single clonal lineage could have colonized the co-located animals and that each premises could represent a transmission cluster.

CDI or asymptomatic carriage

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

CDI was demonstrated by the detection of free toxins, together with GDH, in the two samples of four animals (#3, #32, #36 and #37), which displayed signs of diarrhea (CDL in the colon) and toxigenic *C. difficile* (ribotype 126 or 017) as the only detected entero-pathogen (Table 3, Figure 2). In the remaining sixteen Equidae with a toxigenic *C. difficile*, no free toxins could be detected in any sample (Table 3). Half of them consistently showed no diarrhea signs (#1, #2, #9, #12, #19, #85, #90 and #92). For all remaining animals except one (#57), diarrhea signs could have an independent cause: another entero-pathogen (*C. perfringens* in #43, with *P. sordellii* in #29 and #42 or with both *P. sordellii* and rotavirus in #53, and a parasite in #78) or the intestinal disease that caused death (acute folded colon in #54 and intestinal lymphoma in #81) (Table S1). Finally, sixteen Equidae were asymptomatic carriers of toxigenic *C. difficile,* while possibly having an independent diarrhea.

In the subpopulation displaying toxigenic *C. difficile*, animals with 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 animals with CDI were Trotters and most had received antibiotics (Table S1, Table 1). This showed 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 unrelated to CDI 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).

CDI was the only identified cause of the endo-enterotoxaemia and death of two animals, #32 and #36 (Table S1). Ribotype 017 was implicated in both cases and probably also in another death in 2018 (animal #0, which displayed signs of diarrhea, had received antibiotics and died from endo-enterotoxaemia) (Table 2, Table S1).

Discussion

C. difficile prevalence and diversity

C. difficile prevalence in French necropsied Equidae (27%) was unanticipated by many professionals, probably because its carriage was previously overlooked. Similar prevalence has been reported in European live horses: 4-33% in general and 5-63% for ill animals (9). The dead condition of animals apparently did not result in any major bias, probably because death causes were very diverse (Table S1).

C. difficile strains from Equidae belonged to diverse, already described ribotypes (9, 30). Of note, 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 (31), notably in France (https://www.pasteur.fr/fr/file/44921/download) (30, 32). Most toxigenic ribotypes found in Equidae are very 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 2013-2014 (30). French Equidae therefore contributed to the dissemination of clinically important ribotypes, a preoccupying finding.

Most toxigenic ribotypes evidenced here are also common in animals (9, 11). Ribotypes 126, 020 and 012 had been found in farm animals (9). Ribotype 078, prevalent in animals, notably pigs (9), was rather uncommon here (N=1 isolate), but the phylogenetically close ribotype 126 (33) was represented (N=3) (Table 2). Of note, most toxigenic ribotypes identified here have been detected in diverse sources in Europe, molluscs (005, 012, 017, 020, 078, 126 and AI-53) (34), potatoes (35) and vegetables (012, 020, 078 and 126) (11). The noteworthy diversity and ubiquity of ribotypes identified here highlighted Equidae as important players in the dissemination, circulation and transmission of pathogenic *C. difficile*, a One Health issue.

The co-localization of Equidae sharing the same ribotype (Figure 1) suggested transmission between them, even though the full understanding of *C. difficile* transmission and phylogeny would require further genome-wide investigations. Regarding the co-located carriers of prevalent ribotype 009, described in Czech horses (36), one was a foal born in 2020 (#61), after the death of the others in 2019 (#5, #18, #21) (Table S1), indicating indirect transmission and spore persistence in the stud farm environment for months.

CDI and carriage

To reach a high standard diagnosis of CDI in necropsied Equidae with *post-mortem* signs of diarrhea, we combined the amplification of toxin genes, a method of high sensitivity and negative prediction value, to toxin detection, a method of high specificity and positive prediction value, as recommended in European hospitals (3). Toxin detection is uncommon in animals, notably Equidae (37-39), after *post-mortem* examination (40). Here, toxin detection demonstrated CDI in 4% of the population.

C. difficile carriage concerned 23% of animals. Equine signs of diarrhea or of endo-enterotoxaemia were significantly correlated to *C. difficile* presence (Table S1, Table 1) and

specifically carriage (p-value <0.005). Consistently, more *C. difficile* carriers (n=7/23) than *C. difficile*-negative animals (n=9/73) displayed another entero-pathogen (bacterium, virus, parasite), with a strong trend towards significance (p < 0.06). The most prevalent was *C. perfringens* in four *C. difficile* carriers (#29, #42, #43 and #53) and it was the most probable cause of the endo-enterotoxaemia and death of at least one of them (#43) (Table S1). The co-colonization or co-infection by *C. difficile* and *C. perfringens* have been described in horses (41, 42), dogs (43), pigs (44) and humans (45).

Our most complicated case was animal #29, which displayed three pathogen species and two *C. difficile* strains, toxigenic and non-toxigenic (Table S1, Table 2). Animal co-colonization by a toxigenic strain and a non-toxigenic one of ribotype 009 or 010 has been reported in companion animals (46). Non-toxigenic strains have been shown to prevent CDI or reduce its severity in animal models (47) and, for a ribotype 009 strain, also in commercial piglets (44).

All Equidae with CDI, in contrast to a few asymptomatic carriers of a toxigenic *C. difficile*, were Trotters (Table 1, Table S1). The condition of their microbiota could have promoted CDI. Indeed, most Trotters with CDI had been treated by antibiotics, favoring microbiota dysbiosis (Table 1, Table S1). On the contrary, none of the six Thoroughbred asymptomatically carrying a toxinogenic strain had received antibiotics. Moreover, the four Thoroughbred having received antibiotics carried non-toxinogenic strains of ribotype 009 (Table 1, Table S1), possible able to protect against a toxigenic strain (44, 47).

CDIs in Equidae had been caused by isolates of ribotypes 126 or 017, which were the CloDifEqui isolates least susceptible to vancomycin (MIC=1.0mg/L and 0.75mg/L respectively), and the latter were also poorly susceptible to metronidazole (MIC=0.19-0.38mg/L). This suggested that Equidae treatments against CDIs could select strains more difficult to eradicate and able to better persist.

The ribotypes virulent in Equidae differ by their reported prevalence. Ribotype 126 was the sixth most prevalent ribotype in European patients in 2013-2014 (30) and is also, together with its close relative ribotype 078, prevalent in production animals, notably pigs (9, 48). In Brittany, a French region close to Normandy, biogas plants using porcine, bovine and poultry manure almost exclusively provided ribotypes 078 and 126 (26). Ribotype 017, known as an emerging ribotype in Asia (49), is less frequent in Europe: it was the twelfth most prevalent ribotype in 2013-2014 (30). In France, its prevalence was low in 2011-2014 (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 is also uncommon in animals (9, 49) and has not been detected in Brittany (26), its prevalence in French Equidae (5%) was noticeable. It was responsible for three CDI cases (#32, #36 and #37) in a small area (Figure 1) within six weeks (Table S1), raising the possibility of a local infection cluster. Ribotype 017, whose virulence was of concern, could be emerging in Equidae and/or in Normandy. Finally, French Equidae, as a reservoir for *C. difficile* clinically important ribotypes, would deserve careful monitoring in a One Health perspective.

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Figure Legends

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

Each *C. difficile*-positive Equidae is located on a map of Normandy according to the latitude and longitude coordinates of its premises (Table S1). Different symbols are used depending on *C. difficile* toxin profile: squares for a three-toxin profile (TcdA TcdB and CDT, for ribotypes 078 and 126), circles for two-toxin profile (TcdA and TcdB, for ribotypes 005, 012, 020, 181 and AI-53), triangles for a TcdB profile (for ribotype 017) and finally diamonds for non-toxigenic strains (ribotypes: 009, 035 and 439) (Table 2). *C. difficile* ribotype (Table 2) is indicated in a box, and if several animals of a single premises harboured the same ribotype (Table S1, Table 2), their number is shown between brackets. *C. difficile* pathogenicity is also indicated by a colour code: non-toxinogenic in green, and toxigenic in either orange or red depending on whether *C. difficile* was asymptomatically carried or responsible for a CDI.

Figure 2. Diagnosis of CDI 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 and ribotype of *C. difficile*, and the result of diagnosis, are indicated in four concentric rings.

First (core): *C. difficile* pathogenicity and toxin profile (see 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 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 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): Diagnosis (Table 3). Infection (CDI), asymptomatic carriage of a toxigenic strain or carriage of a non-toxigenic strain are on a red, orange or green background respectively.

Table 1. Equine population: data summary

	***C. difficile* presence**			***C. difficile* pathogenicity**				**Diagnosis** (animals with a 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	59	11	**0.0001**	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	**0.0001**	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	0.83	5	6	0	0.31	3	3	**0.014**
No	43	14		2	11	1		12	0	
Unknown	6	2		0	2	0		1	1	
Previous hospitalization										
Yes	22	11	0.57	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

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 was statistically analyzed according to: i) *C. difficile* presence or not (all animals), ii) *C. difficile* pathogenicity or not (*C. difficile*-positive animals) and finally iii) the diagnosis of CDI or asymptomatic carriage (for positive animals with a toxigenic *C. difficile*).

Table 2. *C. difficile* strains isolated from necropsied Equidae: molecular characterization

Strain Name	Isolated from Equidae n°	Intestinal content	Method Enrichment	Pathogenicity Genotype (PCR) *tpi* [a]	*tcdA* [b]	*tcdB* [a]	lok [c]	*tcdC* [d] deletion	*cdt* [e]	Toxin profile [f]	Type Ribotype [g] (WEBRIBO)	MLST [h]	Antibio-resistance MIC Vancomycin (mg/L) [i]	MIC Metronidazole (mg/L) [i]
2019-2021														
***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	+		**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		**012**	ST54	0.50	0.05
***Cd* E-85**	85	CAE	-									nd	0.50	0.05
***Cd* E-42**	42	CAE	+	+	+	+	-	0	T		**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		**181**	nd	0.38	0.19
***Cd* E-1**	1	CAE	+	+	+	+	-	0	T		**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**
***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	-	+	-	-	+	-	-		**035**	nd	0.13	0.13
***Cd* E-29**	29*	CAE	+	+	-	-	+	-	-		**439**	nd	0.50	0.05
2018														
CdE-0-CDL	0[¤]	CDL	+	+	del	+	-	0	-	TcdB	**017**	nd	0.38	0.032

Table 2 Legend.

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

[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 ('T', i. e. of a pseudogene) or no amplification ('-')

[f] Toxin profile: the combination of toxins encoded on the strain genome (according to the PCR-established genotype)

[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 MIC values are in bold.

[*] animal co-colonized by two different strains (one recovered from CAE and one from CDL)

[¤] animal necropsied at the end of 2018 (pilot study)

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

553 **Table 3. Diagnosis of CDI or carriage**

554

Equidae n°	Intestinal sample	*C. difficile* Strain [a]	Pathogenicity	Toxin profile [b]	Ribotype	Enzymatic Immuno-Assay GDH [d]	Free Toxins [d] (TcdA TcdB)	Diagnosis [d]
3	CAE	*Cd* E-3	**Toxigenic**	TcdA TcdB CDT	126	+	+	**CDI**
	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	-	-	

555
556
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Table 3 Legend.

All data about *C. difficile*-positive Equidae and results are indicated: i) signs of putative diarrhea as inferred from the *post-mortem* observed watery consistency of intestinal sample (CDL, in bold), ii) presence of a *C. difficile* strain and its pathogenicity (toxigenic [in bold] or non-toxigenic strain) and iii) free toxin detection. Animals are grouped according to diagnosis: CDI, asymptomatic carriage of a toxigenic strain or carriage of a non-toxigenic strain.

[a] Strain: preserved strain name or 'not preserved' (a strain that was initially detected but could not be recovered) or 'none' (no detectable *C. difficile*)

[b] Toxin profile, according to the PCR-established genotype of the strain. In one case, '?' is indicated as no PCR targeting *cdt* genes has been performed.

* co-colonized animal

NA: not applicable

ND: not determined

Supporting information

Table S1. Animal data

The registered data of all necropsied Equidae are shown.

Table S2. Sequence Type (MLST) of CloDifEqui strains

The Sequence Type (MLST) of the first isolated strains was characterized.

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

Toxin detection was performed in samples expected to give a negative result as a control for assay specificity (also see Table 3). The samples from *C. difficile*-negative animals that have been assayed are listed here.

Statements

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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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Figure 1

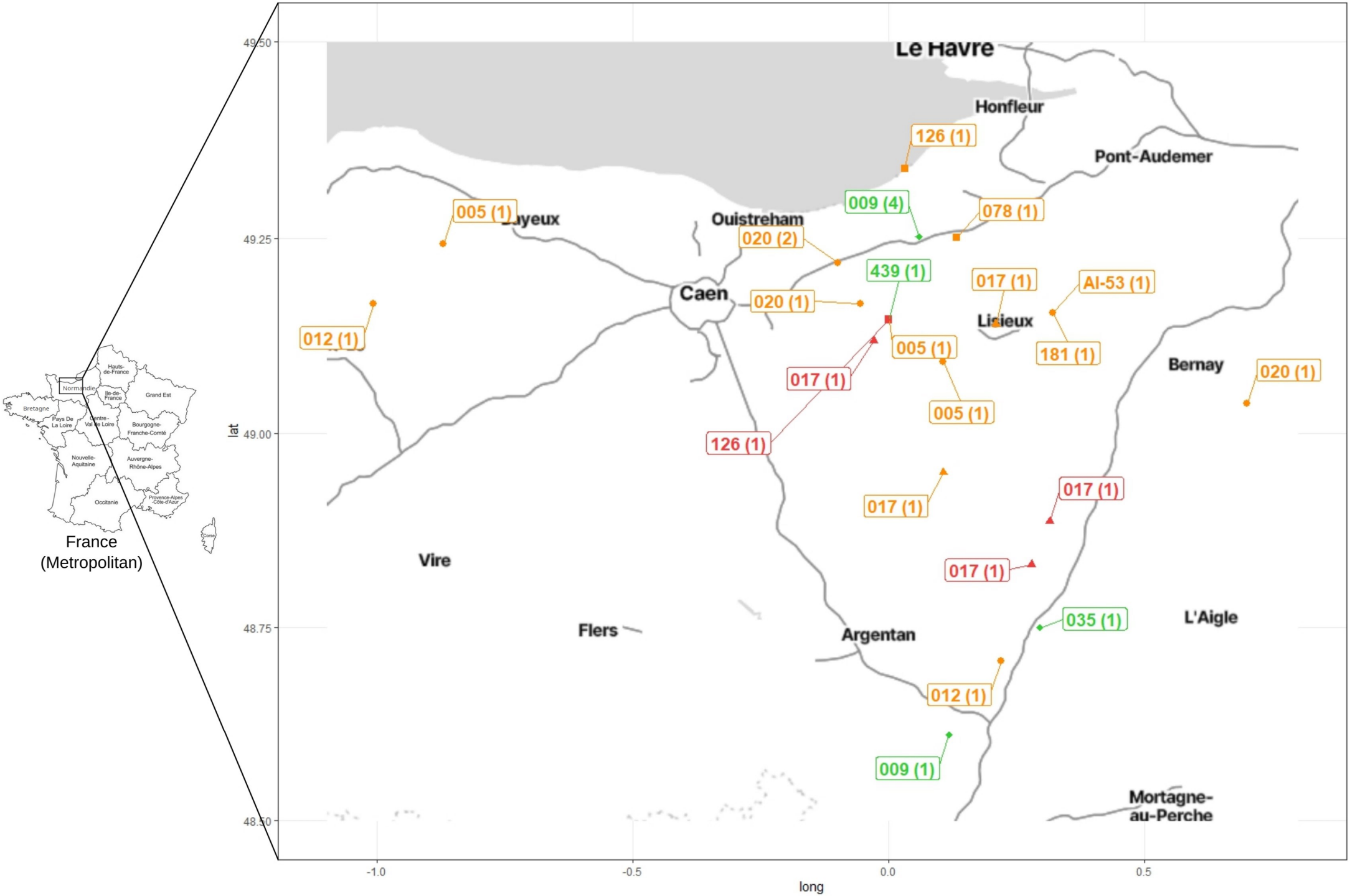
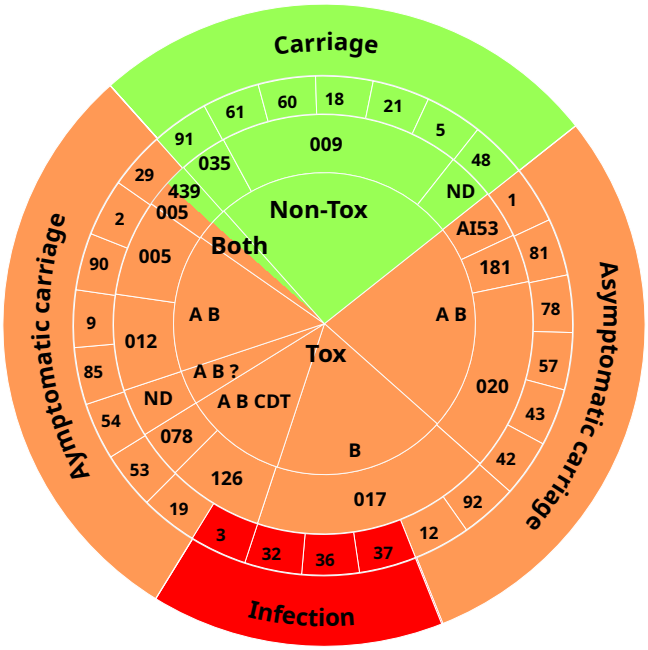

Le Havre
Honfleur
Pont-Audemer
Ouistreham
Caen
Lisieux
Bernay
Vire
Flers
Argentan
L'Aigle
Mortagne-au-Perche
126 (1)
009 (4)
078 (1)
005 (1)
020 (2)
439 (1)
017 (1)
AI-53 (1)
020 (1)
012 (1)
005 (1)
181 (1)
017 (1)
005 (1)
126 (1)
017 (1)
017 (1)
017 (1)
035 (1)
012 (1)
009 (1)
lat
long
49.50
49.25
49.00
48.75
48.50
-1.0
-0.5
0.0
0.5
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