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

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Summary

Clostridioides difficile is an anaerobic, spore-forming entero-pathogen, able to persist in the environment and to cause diarrhea in humans and animals. We took advantage of all necropsies of Equidae in Normandie, France, from 2019 to 2021 (n=100), to systematically recover the digestive content in animal caecum and, if of reduced consistency and possibly diarrheal, in any intestinal segment. 27 Equidae were positive for *C. difficile* and 25 provided 34 isolates forming the first equine strain collection in France (CloDifEqui). Strains were toxigenic in 20 Equidae, including one co-colonized by a non-toxigenic strain. Toxigenic isolates displayed different toxin profiles and belonged to 8 ribotypes (WEBRIBO): 078, 126 (TcdA TcdB and CDT toxins), 005, 012, 020, 181, AI-53 (TcdA and TcdB), 017 (TcdB). Non-toxigenic isolates were of ribotypes 009, 035 and 439. The predominant ribotypes were 017 (n=5 animals, N=8 isolates) and 009 (n=5, N=7). In two premises, a few Equidae shared the same ribotype, either 009 or 020, suggesting two potential transmission clusters. *C. difficile* infection was shown by free toxin detection in 4 animals displaying both *post-mortem* signs of diarrhea (colon watery content) and toxigenic isolates. These isolates belonged to ribotype 126 (n=1) or 017 (n=3) and were the least susceptible to vancomycin. The infection by ribotype 017 was responsible for animal death in two cases. Finally, French Equidae represented a previously underestimated reservoir of *C. difficile* and ribotype 017 was highly virulent in these animals, both preoccupying findings in a One Health perspective.

Introduction

C. difficile is a spore-forming, obligate anaerobe and an entero-pathogen responsible for post-antibiotics diarrhea and colitis (Smits et al., 2016). In the 2000's, it has emerged as a major cause of outbreaks of health-care associated diarrhea in North America and Europe, especially in elderly and/or antibiotics-treated patients. Since the 2010's, it is also known as a rising cause of community-acquired infections. Disease severity and symptom intensity can vary: *C. difficile* can cause mild to severe infections, with complications and frequent recurrences, and can be life threatening. Disease and symptoms are due to *C. difficile* toxins. Both TcdA and TcdB of the Large Clostridial Toxin (LCT) family have cytopathic and cytotoxic effects on epithelial cells in the colon, even though TcdB alone is sufficient to cause disease, and CDT (CdtA-CdtB), the binary toxin, has an accessory role (Orrell and Melnyk, 2021; Kordus et al., 2022). Non-toxigenic strains are unable to cause diarrhea (non-pathogenic), while toxigenic strains can either express virulence (produce their toxins) or be asymptomatically carried (Crobach et al., 2016).

Antibiotics, hospitalization and age are the main risk factors for *C. difficile* infection. Antibiotics notably induce dysbiosis by altering gut microbiota diversity and metabolism, notably with respect to bile salts modification, and thus favor the germination of *C. difficile* spore and the multiplication of vegetative cells, finally leading to toxin production and symptoms. *C. difficile* infections are treated by antibiotics, mostly fidaxomicin and vancomycin whose efficiency is decreasing after each episode, and the last resort treatment of recurrences is fecal microbiota transplant (FMT) (van Prehn et al., 2021). Indeed, antibiotics used against *C. difficile* can, like any other antibiotics, lead to or exacerbate microbiota dysbiosis and if they do not succeed in eradicating the pathogen, they can finally promote a new infectious cycle. *C. difficile* biofilms could contribute to tolerance to antibiotics and to persistence between recurrences (Poquet et al., 2018; Frost et al., 2021; Meza-Torres et al., 2021).

C. difficile, a long-known public health problem, has recently emerged as a major player in One Health (Rodriguez et al., 2016; Rodriguez Diaz et al., 2018; Lim et al., 2020). It has been detected in most animals and is able to infect many of them: wild animals, farm animals including pigs, calves and poultry, and also companion animals (Janezic et al., 2014; Rodriguez et al., 2016; Rodriguez Diaz et al., 2018; Weese, 2020; Bolton and Marcos, 2023). Of note, *C. difficile* strains are able to jump between hosts, leading to cross-species, zoonotic and anthropo-zoonotic transmission (Hensgens et al., 2012; Knetsch et al., 2014). Accordingly, the recent increase in community-acquired infections has been associated to the wide transmission of *C. difficile* strains (Eyre et al., 2013; Ofori et al., 2018; Rodriguez Diaz et al., 2018; Turner et al., 2019), either directly (by contact or consumption of contaminated meat) or indirectly, after fecal contamination of the environment (surfaces, soil, water, cultures, animal hosts and vectors)(Warriner et al., 2017; Lim et al., 2020). The undetectable carriage of toxigenic strains contributes to the spreading of persistent spores in the environment, representing a potential of virulence for any newly contaminated host, including humans.

In this context, *C. difficile* animal hosts are more and more studied (Rodriguez et al., 2016; Rodriguez Diaz et al., 2018). Equidae, which can be racing, sporting, breeding or working animals,

are among the *C. difficile* hosts that have the closest interactions and the most prolonged contacts with humans. Although they are herbivores, they are monogastric mammals like humans. Horses are in general of high value (Institut Français du Cheval et de l'Equitation, https://www.ifce.fr) and their health is usually very well monitored: infections are carefully treated by antibiotics and horse-dedicated health-care centers and hospitals exist. Horses therefore have the same main risk factors for *C. difficile* infections as humans: antibiotic exposure and hospitalization. In both equine and human hosts, *C. difficile* is responsible for the same diseases, diarrhea and colitis, especially post-antibiotics ones, with outbreaks and sporadic cases (Baverud, 2004; Diab et al., 2013; Zhang and Martinod, 2014; Hain-Saunders et al., 2022; Uzal et al., 2022). Both hosts have long been treated by the same antibiotics, metronidazole and vancomycin, until fidaxomicin substituted for metronidazole in the hospital. In horses like in humans, diarrhea can be treated by FMT (Mullen et al., 2018). The main difference between humans and horses as host populations developing *C. difficile* infections is age. Most children of less than three years are asymptomatic carriers of *C. difficile* and in case of diarrhea, *C. difficile* testing, whose positive result is not conclusive, is not recommended (Crobach et al., 2016). On the contrary, *C. difficile* is known to cause infection in foals, not only in the colon as in equine adults, but also in the small intestine (Baverud, 2004; Diab et al., 2013).

Although *C. difficile* importance in animals and in particular horses is raising more and more interest (Rodriguez Diaz et al., 2018; Lim et al., 2020; Hain-Saunders et al., 2022; Bolton and Marcos, 2023), data remain scarce in France. Diarrhea has several bacterial and viral etiologic agents, without any specific pathognomonic signs, and *C. difficile* testing is not always performed. Moreover, bacteriological analyses used to focus on aerobic pathogens because many laboratories lacked anaerobic growth equipment. Finally, in case of diarrhea, the detection of a *C. difficile* toxigenic strain is not sufficient to diagnose a *C. difficile* infection, as asymptomatic carriage cannot be excluded (Crobach et al., 2016).

Here, we decided to study *C. difficile* in Equidae necropsied in Normandie, the first breeding region in France (https://www.ifce.fr). Compared to most studies of *C. difficile* in animals, here, animal necropsy (Tapprest et al., 2019) was a double opportunity. It first facilitated recovering digestive contents from a diverse population showing very different clinical pictures. It also gave access to all animal data registered before, during and after necropsy. They include: i) individual data (sex, age, breed, geographic location), ii) clinical data and possible *ante-mortem* treatments, iii) *post-mortem* observations and results (anatomo-pathology examination of tissues and lesions, complementary analyses including microbial and molecular searches for infectious agents) (Duchesne et al., 2019), and finally iv) the cause of animal death, resulting from the analysis and synthesis of all epidemiological, clinical and *post-mortem* data.

Our study was aimed to evaluate i) *C. difficile* occurrence, diversity and putative circulation among French Equidae, and ii) the prevalence of *C. difficile* infection or carriage after a *post-mortem* diagnosis including free toxin detection.

Results

Equine necropsies and sampling

Equine necropsies in Normandie from 2019 to 2021

Equidae necropsies are frequent and performed at the request of their breeders or veterinarians, in order to determine the cause of animal death. Here, we included the whole equine population (except fetus) necropsied by ANSES in Normandie, from May 2019 to August 2021, without any selection, neither on animal clinical picture nor on death cause. During this period, Covid-2019 and the consecutive lockdowns in 2020 and 2021 were milestones that negatively affected all human activities. Necropsy activity was clearly decreased compared to previous years: at the end of the study, after more than two years, 100 necropsies had only been performed in total (Table S1), barely corresponding to previous annual average (100 to 150 necropsies per year in the 2005 - 2017 period).

Population of necropsied Equidae

Almost all necropsied Equidae except three originated from Normandie (Table S1). The whole equine population (Table 1) was mainly composed of females (67%) and of <1-year-old animals (59%), including 48% of foals (less than 6 months) and 11% of yearlings (from 6 months to less than 1 year). The whole population comprised nine breeds, with most animals being Thoroughbred (42%), Trotters (31%) and Saddlebred horses (11%; Table 1). The cause of animal death remained unknown in only few cases while being established for most of the population. In some cases, the *post-mortem* picture was complex and animal death was attributed to combined pathologies or infections (Table S1). In most cases however, death was due to a single cause among diverse pathologies, including non-infectious ones (e.g. myopathy, intestinal diseases like volvulus or intussusception…), infections (e. g. systemic, respiratory, intestinal infections …) or trauma (e.g. perforations, hemorrhages, fractures). The most prevalent cause of animal death was endo-enterotoxaemia (12%), which is characterized by toxin-mediated intestinal inflammation, and can be caused by toxin-producing clostridial species like *C. difficile*, *Clostridium perfringens* and *Paeniclostridium sordelli* (Uzal et al., 2022) or by endotoxin (LipoPolySaccharide)-producing Gram-negative bacteria like *Salmonella enterica*. The second most prevalent death cause was infection by *Rhodococcus equi* (9%), a major and mainly respiratory pathogen and a common cause of death for foals (Duquesne et al., 2017; Sanz, 2023).

Systematic sampling of digestive contents

Digestive contents were systematically collected from the gut of all animals. Sampling was performed in at least one and up to two intestinal segments: i) the caecum (CAE), in all cases, and ii) any other segment whose content was observed to be of reduced, watery consistency ('CDL' for watery digestive content in French), which could represent a *post-mortem* sign of diarrhea. A single sample, that from the caecum (CAE), or two (CAE and CDL) were respectively recovered from 70 and 30 animals, but in one animal, only one of the two recovered samples (CDL) was preserved. This led to a total of 129 fecal samples, 99 CAE, and 30 CDL mostly from animal colon (Table S1).

C. difficile prevalence and characterization of the *C. difficile*-positive equine subpopulation

Systematic search for *C. difficile*

C. difficile was systematically searched in all intestinal samples. This was performed directly on commercial screening agar plates, or, if this did not provide any candidate, after multiplication in a rich liquid medium. In all cases, a primary molecular screening of *C. difficile* candidates was performed by standard PCRs targeting *tpi*, a house-keeping gene marker of *C. difficile,* together with *tcdA* and *tcdB,* the main toxin genes (Lemee et al., 2004; Persson et al., 2011). Finally, in most cases, the species of either one candidate colony or a mix of a few candidate colonies was also confirmed by Matrix-Assisted Laser Desorption/Ionization Time-Of-Flight (MALDI-TOF) mass spectrometry.

C. difficile was detected in 36 fecal samples recovered from 27 animals. These samples were the single CAE sample of 11 animals, one of the two samples of 7 animals and finally the two samples of 9 animals (Table S1, Table 1).

Composition of the *C. difficile*-positive equine subpopulation

The presence of *C. difficile* in Equidae did not significantly vary according to animal sex, age, breed, previous antibiotic treatment or hospitalization (Table 1). On the contrary, it was significantly correlated to the number of intestinal samples per animal: two samples (CAE and CDL) had been recovered from more *C. difficile*-positive Equidae (59%) than negative ones (18%) (Table 1). The presence of *C. difficile* in an animal was also significantly correlated to its *post-mortem* signs of endo-enterotoxaemia: intestinal tissue inflammation, consistent with toxin activity, had more often been observed in the *C. difficile*-positive subpopulation than in the negative one (48% vs 9.5%) (Table 1).

Strain preservation as a collection

C. difficile isolates were preserved immediately after having been confirmed by PCR (Lemee et al., 2004; Persson et al., 2011), except in two cases: a non-toxigenic strain and a toxigenic one, initially detected in animals #48 and #54 respectively, subsequently failed to be isolated again despite independent assays. In all other cases, one isolate per positive sample was preserved, leading to up to two isolates per animal. A single isolate was obtained from sixteen animals, while a pair of isolates were collected from nine animals (Table S1, Table 2). In total, this led to 34 isolates from 25 Equidae necropsied from 2019 to 2021: they were named '*Cd*E' (for *C. difficile* from Equidae) followed by an animal identification number and when necessary, 'CDL' (Table 2). An additional isolate had been obtained during a preliminary set-up of *C. difficile* search. At the end of 2018, five Equidae were selected because of their *post-mortem* signs of endo-enterotoxaemia and watery intestinal contents, and the colon watery content of one of them (#0) provided our first *C. difficile* isolate (*Cd*E-0-CDL). Finally, in this study, we assembled the first library of *C. difficile* strains originating from Equidae in France, named CloDifEqui (Table 2).

198 ***C. difficile* strain molecular diversity**

199 Toxin genes

200 The toxin gene profile of all preserved isolates was established by a multiplex PCR (*tcdA*, *tcdB*,
201 *tcdC, cdt, lok*) (Le Maréchal et al., 2020). It revealed: i) wild type-size or truncated *tcd* and *cdt* alleles,
202 including *tcdC* alleles with in-frame deletions characterizing epidemic and clinically important ribotypes
203 (Bouvet and Popoff, 2008; Goorhuis et al., 2008) or ii) the absence of *tcd* genes (deletion of the whole
204 Pathogenicity Locus, *lok*) (Braun et al., 1996).

205 Nine *Cd*E isolates were non-toxigenic (Table 2) and originated from 7 animals, including six
206 Thoroughbred (Table S1). The remaining twenty-five toxigenic *Cd*E isolates could be grouped into
207 different toxin profiles according to the toxins they were able to produce: i) all three toxins (TcdA TcdB
208 and CDT) (N=4 isolates), ii) the two main toxins (TcdA and TcdB) (N=13) and iii) a single toxin (TcdB)
209 (N=8, Table 2). They originated from 19 animals of six different breeds (Table S1). When considering
210 all *C. difficile*-positive animals, even if their strain had not been preserved, 20 animals displayed at
211 least one toxigenic strain able to cause disease (Table S1, Table 2).

212 We examined the pathogenicity of the two isolates recovered from the same animal. For almost
213 all pairs, both isolates shared the same pathogenicity profile (Table 2). Both were toxigenic and able
214 to produce the same toxins in six animals (TcdA TcdB and CDT for strains of animal #3, TcdA and
215 TcdB for strains of animals #42 and #43 and TcdB for strains of animal #32, #36 and #37) and both
216 were non-toxigenic in two animals (#18 and #60). On the contrary, the last animal (#29) was co-
217 colonized by a toxigenic strain in the intestine (TcdA and TcdB profile) and a non-toxigenic one in the
218 caecum (Table 2).

219

220 Antimicrobial susceptibility

221 Equine strains were studied for their susceptibility to metronidazole and vancomycin used to
222 treat *C. difficile* infection in horses. All strains were found to be susceptible to each antibiotic (Table
223 2). For metronidazole, strain Minimum Inhibitory Concentrations (MICs) ranged from 0.03 to 0.38 mg/L,
224 with a MIC50 (MIC value for half isolates) of 0.13 mg/L. The highest MIC values (from 0.19 mg/L to
225 0.38 mg/L) were displayed by strains of only two ribotypes: 017 (the seven strains from animals #32,
226 #36, #37 and #92) and to a lesser extent, 020 (three strains from animals #42 and #43) (Table 2). For
227 vancomycin, strain MICs ranged from 0.13 to 1.0 mg/L, with a MIC50 of 0.5 mg/L. The highest MIC
228 value (1 mg/L) was displayed by the two strains of ribotype 126 recovered from animal #3. The second
229 highest MIC value (0.75 mg/L) was shared by one third of strains, mostly belonging to ribotype 017
230 and to a lesser extent, to ribotypes 009 or 005 (Table 2). Of note, all pairs of ribotype 017 strains
231 originating from the same animal (#32, #36 and #37) showed both the highest levels of metronidazole
232 resistance (MICs ranging from 0.19 mg/L to 0.38 mg/L) and the second highest level of vancomycin
233 resistance (MIC of 0.75 mg/L, Table 2).

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PCR-ribotyping

All strains were characterized by PCR-ribotyping (Ferraris et al., 2019), and the first isolated ones also by Multi Locus Sequence Typing (MLST) (Griffiths et al., 2010), with consistent results (Table S2, Table 2). The CloDifEqui isolates were representative of eleven ribotypes (Table 2) belonging to four clades (1, 2, 4 and 5).

Toxigenic isolates belonged to eight PCR ribotypes assigned according to the WEBRIBO database (WRT). The isolates with a three-toxin profile were of WRT126 (N=3) and WRT078 (N=1). The isolates endowed with a TcdA and TcdB profile belonged to WRT020 (N=6), WRT005 (N=3), WRT012 (N=2), WRT181 (N=1) and WRTAI-53 (N=1). The isolates with a TcdB profile were of WRT017 (N=8) (Table S2, Table 2). Non-toxigenic isolates were assigned to three WRTs: WRT009 (N=7 isolates), WRT035 (N=1) and WRT439 (N=1) (Table S2, Table 2).

We analyzed the conservation of ribotypes between the two isolates of the same animal. When they shared the same pathogenicity profile and the same toxin profile, they also shared the same ribotype, suggesting that they could be phylogenetically close and raising the possibility that they could belong to a single clonal lineage present in the animal gut. On the contrary, the non-toxigenic and toxigenic isolates of animal #29 belonged to different ribotypes (WRT439 and WRT005), further supporting a model of animal co-colonization by two phylogenetically distant strains (Table 2).

The distribution of ribotypes in the equine population was then studied. The two most frequent ribotypes (each in n=5 animals) were WRT017 (animals #12, #32, #36, #37 and #92, N=8 isolates) and WRT009 (animals #5, #18, #21, #60 and #61, N=7 isolates). They were followed by WRT020 (n=4, N=4), WRT005 (n=3, N=3), WRT126 (n=2, N=3) and WRT012 (n=2, N=2). Of note, our first obtained strain (*Cd*E-0-CDKL), was also of ribotype 017 (Table 2).

Putative circulation of *C. difficile* between animals: an epidemiological analysis at the ribotype level

In our study, all *C. difficile*-positive Equidae belonged to premises located in a relatively small area of ~ 50 km x 50 km in Normandie (Table S1, Figure 1). *C. difficile* putative circulation among Equidae was studied by cross-analyzing strain ribotypes and animal locations. In two cases, a few *C. difficile*-positive animals of a single premises, in contact to the same environment and possibly with each other, shared strains of a single ribotype. First, in a stud farm between Hérouville-Saint-Clair, Trouville-sur-Mer and Pont-Audemer (Figure 1), four Equidae whose necropsies took place first in 2019 during a one-month period (#5, #18 and #21) and then one year later, in 2020 (#61), all carried non-toxigenic strains of WRT009. Second, in another premises between Ouistreham and Hérouville-Saint-Clair (Figure 1), two Equidae (#42 and #43), which had been necropsied within a few days in 2019, displayed toxigenic strains of WRT020. As strains of the same ribotype should be phylogenetically close, our results suggested that in each case, the same clonal lineage could have disseminated and contaminated several animals and that each premises could represent a cluster of *C. difficile* circulation and transmission.

276 ***C. difficile* infection or not**

277 *Post-mortem* detection of toxins

278 An important question was whether *in vivo,* in Equidae (*ante-mortem*), *C. difficile* had been
279 able to produce its virulence factors. The presence of free toxins in animal intestinal samples was
280 tested using a commercial enzymatic immuno-assay against TcdA and TcdB. In order to check the
281 assay specificity when using equine digestive contents rather than human stools, we included samples
282 expected to give a toxin-negative result according to available data: i) all samples from animals
283 displaying a non-toxigenic *C. difficile* (Table 3) and ii) many samples devoid of any detectable
284 *C. difficile*, including all samples from animals with endo-enterotoxaemia signs and many CDL
285 samples (Table S3). No toxins could be detected in any of these samples, while *C. difficile* glutamate
286 dehydrogenase (GDH) was positive in most of the former ones (Table S3, Table 3), showing the assay
287 to be of reliable specificity even when applied to equine digestive contents.

288

289 *C. difficile* infection or asymptomatic carriage of a toxigenic *C. difficile*

290 All intestinal samples of Equidae displaying signs of diarrhea and a toxigenic *C. difficile* were
291 tested. In the case of 4 animals with a colon watery content (#3, #32, #36 and #37), free toxins,
292 together with GDH, were detected in both animal samples (Table 3, Figure 2), which also contained
293 toxigenic isolates of the same ribotype: WRT017 (for #32, #36 and #37) or WRT126 (for #3) (Table 3,
294 Figure 2). This demonstrated *C. difficile* infection in all cases.

295 Free toxins could not be detected in any sample from the remaining Equidae with signs of
296 diarrhea and a toxigenic *C. difficile* (#29, #42, #43, #53, #54, #57, #78 and #81). Neither could they
297 be detected in animals with a toxigenic *C. difficile* but no signs of diarrhea (#1, #2, #9, #12, #19, #85,
298 #90 and #92). All these 16 Equidae were asymptomatic carriers of a toxigenic *C. difficile* (Table 3).
299 Despite the absence of *C. difficile* toxins in their intestinal samples, seven of them displayed *post-*
300 *mortem* signs of endo-enterotoxaemia (#19, #29, #42, #43, #53, #78 and #81) (Table S1). Independent
301 bacteriological analyses identified other toxin-producing entero-pathogens: *C. perfringens* alone (in
302 animal #43) or with *P. sordelli* (in #29, #42 and #53; Table S1). Either species or both could be
303 responsible for animal endo-enterotoxaemia and even for its death in two cases (#42 and #43) (Table
304 S1).

305

306 Equidae with a *C. difficile* infection compared to asymptomatic carriers of a toxigenic *C. difficile*

307 In the population of Equidae displaying a toxigenic *C. difficile*, animals with a *C. difficile*
308 infection and asymptomatic carriers were similar according to animal sex, age, or non-pathognomonic
309 signs of endo-enterotoxaemia or diarrhea (Table 1). On the contrary, *C. difficile* infection compared to
310 asymptomatic carriage of a toxigenic *C. difficile* was statistically correlated to two parameters: animal
311 breed and previous antibiotic treatment (Table 1). First, concerning animal breed, all animals with a *C.*
312 *difficile* infection, but only a minority of asymptomatic carriers, were Trotters (Table 1, Table S1).
313 Second, antibiotics had previously been given to most animals with a *C. difficile* infection, but only to

314 a minority of carriers (Table S1, Table 1). Antibiotic treatment of the Equidae under study was thus
315 shown to represent a risk factor for *C. difficile* infection, as expected.
316
317 *C. difficile* infection as the cause of animal death or not
318 We wondered whether *C. difficile* infection could have been responsible for animal death and
319 therefore examined the cause of animal death in the four cases of *C. difficile* infection (Table S1). In
320 two of them, death was attributed to a cause independent of *C. difficile*. First, a severe intestinal
321 pathology, the intussusception of small intestine, was responsible for the death of animal #3, which
322 was fulminant (animal #3 was a 15 days-old foal; Table S1), and might have hidden or precluded full
323 signs of endo-enterotoxaemia (Table S1, Table 3). Second, the death of animal #37 was attributed to
324 multiple infectious causes, according to *post-mortem* observed signs of respiratory, musculoskeletal
325 and digestive infections and of endo-enterotoxaemia, and to the identification of *Klebsiella*
326 *pneumoniae* in lungs, lung abscess and hock muscle (Table S1). According to available data,
327 *K. pneumoniae* and *C. difficile* could be responsible for the respiratory and musculoskeletal infections
328 and for the intestinal infection, respectively.
329 *C. difficile* infection was responsible for the death of the last two animals (#32 and #36): i)
330 endo-enterotoxaemia, whose signs had been observed during necropsy, was the only identified cause
331 of animal death, ii) after bacteriological search for both aerobic and anaerobic species, *C. difficile* was
332 the only detected pathogen in intestinal samples (Table S1), which also contained its toxins (Table 3).
333 The prevalence of *C. difficile* infection as the cause of animal death in the equine population from 2019
334 to 2021 was therefore of 2%, in good agreement with ANSES previous data. Of note, the death of
335 animal #0 in 2018, which had been attributed to endo-enterotoxaemia, was also probably due to a
336 *C. difficile* infection by a strain of ribotype 017: even though free toxin detection had not been
337 performed, animal #0 showed *post-mortem* observed signs of endo-enterotoxaemia and a colon
338 watery content containing *C. difficile* as the only identified entero-pathogen, and it had previously been
339 treated by antibiotics.

340 **Discussion**

341 ***C. difficile* prevalence in necropsied Equidae**

342 We chose Equidae as a model for animal hosts of *C. difficile*, and studied necropsied Equidae
343 whose registered data are as detailed as those of patients. We systematically included all 100 Equidae
344 necropsied at ANSES in Normandie from 2019 to 2021, without any selection neither on clinical picture
345 nor on death cause. *C. difficile* prevalence in the population under study was of 27%. Equidae
346 contamination by *C. difficile* in Normandie, which used not to be evaluated, seemed to have usually
347 been under-estimated, as such a high level was not anticipated by most professionals in the region.
348 And yet, it was of the same order of magnitude as the previously published prevalence in European
349 live horses, which ranged from 4-33% when considering all animals independently of health or
350 disease, to 0-10% in healthy animals and 5-63% in ill animals (Rodriguez Diaz et al., 2018). This
351 finding suggested that the dead condition of animals had not resulted in any major bias in our study,
352 probably because the Equidae studied here died from very diverse causes, of which endo-
353 enterotoxaemia represented only 12% (Table S1).

354 Interestingly, much more *C. difficile*-positive animals than and negative ones showed i) signs
355 of diarrhea, as inferred from their watery intestinal content (CDL) or ii) signs of endo-enterotoxaemia
356 (Table S1). These statistically significant observations (Table 1) suggested that *C. difficile* could be
357 more common in Equidae with intestinal disorders or diseases. Giving some support to this hypothesis,
358 the death of a higher proportion of *C. difficile*-positive animals (10/27, 37%) than negative ones (12/73,
359 16%) was attributed to a bacterial intestinal infection, with a strong trend toward significance (P= 0.054)

360

361 **Diversity of *C. difficile* strains isolated from Equidae**

362 *C. difficile* strains isolated from Equidae were quite diverse and belonged to the four main
363 clades and eleven ribotypes. Most ribotypes identified here in Equidae are quite common, while 439
364 and AI-53 had much less frequently been observed (Freeman et al., 2018; Rodriguez Diaz et al.,
365 2018). Of note, no representative strain of the historical and epidemic ribotype 027, widely known as
366 responsible for outbreaks in North America and Europe in the 2000's (Smits et al., 2016), was identified
367 in the equine population under study. 027 strains had been described in horses, but rather in the
368 2010's (Hain-Saunders et al., 2022). This might be correlated to the overall change in *C. difficile*
369 epidemiology and the decreased prevalence of 027 strains in patients since the 2010's (Turner et al.,
370 2019), and notably in France (National Reference Laboratory Report, 2020
371 https://www.pasteur.fr/fr/file/44921/download) (Freeman et al., 2018; Le Monnier et al., 2022).

372 Most ribotypes evidenced in Equidae have also previously been detected in many animals like
373 farm animals and horses (Rodriguez Diaz et al., 2018). The most frequent ribotype of equine non-
374 toxigenic strains, 009, has also previously been described in Czech horses (Kecerova et al., 2019)
375 and companion animals (Alves et al., 2023). Ribotypes 078, 126, 020 and 012 had frequently been
376 found in farm animals and in meat, and notably for 078 in pork, beef and chicken meats (Bolton and
377 Marcos, 2023). Of note, ribotype 078, which has widely been characterized in animals, mostly pigs but
378 also horses (Rodriguez Diaz et al., 2018), was rather uncommon in the equine population under study

379 (N=1 isolate). However, three isolates of WRT126 could also be obtained here from necropsied
380 Equidae, and ribotypes 078 and 126 are well-known to be phylogenetically close (Stabler et al., 2012;
381 Schneeberg et al., 2013). The four strains belonging to WRT078 and WRT126 were consistently the
382 only CloDifEqui strains to display a 39 bp-deletion in *tcdC*, which had first been evidenced in strains
383 of ribotype 078 and then of ribotype 126 (Bouvet and Popoff, 2008; Goorhuis et al., 2008). Of note, in
384 Bretagne, a close French region and an important region for pig breeding, the manure of animals,
385 mostly pigs (alone or together with either bovine or both bovine and poultry), but also bovine and
386 poultry, collected in five biogas plants, provided *C. difficile* isolates of almost exclusively ribotypes 078
387 and 126 (Le Maréchal et al., 2020). Here, in Equidae, *C. difficile* ribotypes were much more diverse,
388 possibly because of host differences and/or breeding conditions.
389 Moreover, most ribotypes found in Equidae have also been detected in many environmental
390 sources. For example, common ribotypes, like 005, 009, 020, 012, 017, 078 and 126, but also rare
391 ones, like AI-53 and 439, have been detected in marine bivalve shellfish and mollusks, which represent
392 both a reservoir of *C. difficile* strains and a vector of dissemination (Pasquale et al., 2012; Agnoletti et
393 al., 2019). Ribotypes 020, 012, 078 and 126 have also been found in potatoes (Tkalec et al., 2019;
394 Tkalec et al., 2022) and vegetables, like salads and lettuce (Bolton and Marcos, 2023). 035 strains
395 have recently been evidenced in soil samples in Iraq (Rashid et al., 2023). Finally, all ribotypes found
396 here in Equidae seem to be largely present in the environment and different sources, in agreement
397 with the importance of environmental dissemination in *C. difficile* epidemiology (Warriner et al., 2017;
398 Lim et al., 2020).
399
400 **Circulation and putative transmission of *C. difficile* ribotypes among the equine population**
401 Two putative clusters of *C. difficile* dissemination and transmission could be identified. In a
402 stud farm, four animals all carried non-pathogenic strains of ribotype WRT009, suggesting that
403 phylogenetically close strains could have been transmitted among animals. Of note, the necropsy of
404 the last animal, a 2.5 months-old foal, took place one year later than the other ones, suggesting the
405 persistence of WRT009 in the stud farm environment for several months. *C. difficile* spores in general
406 are long known as highly resistant to stresses and notably to oxygen (Edwards et al., 2016) and
407 responsible for airborne (Deakin et al., 2012) and environmental transmission (Warriner et al., 2017).
408 And strains of ribotype 009 have recently been found to persist in recreational sandboxes for children
409 and dogs in Spain (Orden et al., 2018).
410 In another premises, two animals (#42 and #43) shared toxigenic strains of ribotype WRT020
411 and were shown to be *C. difficile* asymptomatically carriers (Table 3). No evidence for any premises
412 being a transmission cluster of virulent strains had been obtained here, as all three animals having a
413 *C. difficile* infection caused by the same ribotype, WRT017 (#32, #36 and #37) were from different
414 stud farms (Figure 1). These three cases could represent sporadic cases or alternatively, a local
415 outbreak. In support to the latter hypothesis, they occurred in a small area in Normandie (Figure 1),
416 during a relatively short period of ~6 weeks (according to necropsies dates, Table S1), and after a
417 relatively recent contamination in at least two of them (as inferred from animal ages of 3 and 3.5

418 months, Table S1). Finally, the full understanding of *C. difficile* strain circulation and transmission
419 between animals would deserve further investigation and require a genome-wide epidemiological
420 analysis.
421
422 **Prevalence, diversity and asymptomatic carriage of *C. difficile* toxigenic strains**
423 20% of Equidae necropsied in Normandie from 2019 to 2021 displayed at least one *C. difficile*
424 toxigenic strain. Such a high prevalence of toxigenic strains in the whole equine population is
425 preoccupying. The majority of these animals (16% of the whole population under study) were
426 asymptomatic carriers that would have remained undetected in the absence of our study, while
427 contributing, like animals with a *C. difficile* infection, to the dissemination of toxigenic spores.
428 Furthermore, almost all toxigenic strains originating from Equidae belonged to ribotypes that are
429 common in patients. Indeed, ribotypes 020, 078, 126, 005 and 017 were among the Top15 ribotypes
430 found in European patients in 2013, respectively occupying the fourth, the fifth, the sixth and the twelfth
431 rank (Freeman et al., 2018). Our study strongly suggests that Equidae in France could play a role in
432 the dissemination of *C. difficile* pathogenic strains and their transmission to other hosts, including
433 humans.
434
435 **Presence of several entero-pathogens altogether**
436 A possible cause for endo-enterotoxaemia signs in asymptomatic carriers of a toxigenic
437 *C. difficile* could be identified in four cases (#29, #42, #43 and #53): *C. perfringens* (in animal #43) and
438 either *C. perfringens* or *P. sordellii* or both (in the three other cases), even though their pathogenicity
439 has not been established (Zakia et al., 2023). Of note, *C. perfringens,* alone or together with
440 *Salmonella enteritis*, an endotoxin-producing bacterium, could also have caused the endo-
441 enterotoxaemia of *C. difficile*-negative animals (Table S1).
442 The concomitant presence of one or more pathogens together with *C. difficile*, which
443 highlighted the complex clinical picture of some necropsied animals, was not unprecedented. Co-
444 colonization or co-infection by both *C. difficile* and *C. perfringens* had previously been described in
445 foals (Uzal et al., 2012), healthy horses (Schoster et al., 2012), dogs (Diniz et al., 2018) and piglets
446 (Oliveira Junior et al., 2019b). Moreover, co-infection by toxigenic *C. difficile* and *C. perfringens* has
447 previously been described to occur at high frequency in diarrheic patients in Colombia (Forero et al.,
448 2019). Moreover, a recent analysis of the microbiomes of healthy people and ill patients of different
449 ages revealed a different microbiome composition between *C. difficile* carriers and *C. difficile*-negative
450 healthy people (Ferretti et al., 2023). The microbiome of the former subpopulation was enriched in
451 pathogen species, including *C. perfringens*, while commensal species were reduced. This suggested
452 asymptomatic carriage to be an intermediate state between disease and full health in the pathogen
453 absence (Ferretti et al., 2023).
454
455
456

457 **Co-colonization by both a toxigenic strain and a non-toxigenic one**

458 Among *C. difficile* asymptomatic carriers with several entero-pathogens, the most complicated
459 case was that of animal #29. This animal not only displayed three toxin-producing clostridial species,
460 but also two different *C. difficile* strains. Animal #29 could have had an infection by *C. perfringens*
461 and/or *P. sordellii*, leading to its signs of diarrhea and endo-enterotoxaemia (Table S1), while being
462 asymptomatically carrying a toxigenic WRT005 strain in the intestine and a non-toxigenic WRT439
463 isolate in the caecum (Table 2 and 3).

464 Animal co-colonization by both a toxigenic isolate and a non-toxigenic one of a ribotype
465 evidenced here in Equidae has already been described. For example, strains of ribotype 009 have
466 recently been found together with toxigenic strains in companion animals (Alves et al., 2023).
467 Moreover, non-toxigenic strains have been reported to prevent or reduce clinical signs of *C. difficile*
468 infection in animal models, like laboratory rodents and piglets (Gerding et al., 2015; Arruda et al., 2016;
469 Gerding et al., 2018). A strain of ribotype 035 had been found to be part of the normal flora of a mouse
470 colony and to partially protect it from a challenge by a pathogenic strain, significantly decreasing
471 disease severity (Maslanka et al., 2020). Z31, a non-toxigenic strain of ribotype 009, has been shown
472 not only to prevent *C. difficile* infection in experimentally challenged model piglets (Oliveira Junior et
473 al., 2019a) but also to reduce the occurrence of *C. difficile* infection in one-day-old piglets of a
474 commercial farm (Oliveira Junior et al., 2019b). Of note, after Z31 administration, the isolation of *C.*
475 *perfringens* in this piglet population also seemed to be reduced (Oliveira Junior et al., 2019b). Finally,
476 a *C. difficile* non-toxigenic member of WRT439 has been evidenced here altogether with a toxigenic
477 strain in the same animal for the first time to our knowledge. There is no evidence that WRT439 strain
478 might have prevented infection of animal #29 by WRT005 strain, but it had not prevented its endo-
479 enterotoxaemia probably by *C. perfringens* and/or *P. sordellii*.

480

481 **Equidae with a *C. difficile* infection and risk factors**

482 To diagnose *C. difficile* infection. in necropsied Equidae, we combined toxin detection, a
483 method of high specificity and positive prediction value, to the amplification of toxin genes, a method
484 of high sensitivity and negative prediction value, to reach a high standard diagnosis of *C. difficile*
485 infection, as recommended in European hospitals (Crobach et al., 2016; Czepiel et al., 2019). Toxin
486 detection is rare in animals, even though it has previously been performed in Equidae (Arroyo et al.,
487 2007; Ossiprandi et al., 2010; Magdesian and Leutenegger, 2011), including after *post-mortem*
488 examination (Zakia et al., 2023). Here, *C. difficile* infection could be demonstrated by toxin detection
489 in necropsied Equidae with *post-mortem* signs of diarrhea and a toxigenic *C. difficile*, with a prevalence
490 of 4% of the whole equine population under study.

491 Of note, Equidae with a *C. difficile* infection significantly differed from asymptomatic carriers of
492 a toxigenic *C. difficile* according to breed. All the former, but only a minority of the latter, were Trotters.
493 As Thoroughbred horses, like Japanese Thoroughbred horses for example (Uchida-Fujii et al., 2023),
494 can suffer *C. difficile* infections, our finding unexpectedly pointed out to Trotters as highly susceptible
495 to *C. difficile* infections. Several hypotheses, possibly in combination, might explain this result. First, it

496 cannot be excluded that Trotters might have some genetic predisposition favoring *C. difficile* infections,
497 although there is no particular evidence that this might be the case. And even, on the opposite, one
498 could have expected Thoroughbred horses, whose inbreeding level is high, to be more susceptible to
499 infections in general. Second, some breeding conditions might have favored Trotters contamination.
500 For example, French Trotters may be kept in collective housings, favoring contacts with the manure
501 of other animals, while the most valuable Thoroughbred horses almost systematically have individual
502 boxes, probably leading to better overall conditions of infection containment. Third, *C. difficile*
503 infections in Trotters could indirectly result from the condition of their microbiota. Antibiotic treatments,
504 which are well-known to promote microbiota dysbiosis and have been confirmed to be a major risk
505 factor for *C. difficile* infection in French necropsied Equidae (Table 1), have been given to more
506 *C. difficile*-positive Trotters (3 out of 8) than *C. difficile*-positive Thoroughbreds (4 out of 12; Table S1).
507 In addition, a non-toxigenic strain, which might have a protective effect against a newly contaminating
508 toxigenic strain, was carried by only a minority of *C. difficile*-positive Trotters (2 out of 8), but half (6
509 out of 12) of *C. difficile*-positive Thoroughbred (Table S1).
510
511 **Virulence of WRT126 and WRT017**
512 *C. difficile* infections in Equidae had been caused by WRT126 and WRT017 isolates. Of note,
513 these isolates, compared to all other ones in CloDifEqui, were among the least susceptible to
514 vancomycin, and for those of WRT017, also the least susceptible to metronidazole. This finding
515 suggested that treatments against *C. difficile* infections in Equidae in Normandie could select strains
516 both more difficult to eradicate and able to better survive in case of a new infectious cycle and
517 treatment.
518 Strains of ribotype 126 are known as main causes of *C. difficile* infections in patients in Europe.
519 Ribotype 126 was among the Top10 most prevalent ribotypes in Europe in a 3-year period from 2011
520 to 2014, and the sixth one in 2013-2014, with a prevalence of 5.1% (Freeman et al., 2018).
521 Ribotype 017 has mostly been described as an emerging ribotype in Asia in the past few years
522 (Imwattana et al., 2019), and as a relatively common ribotype in Europe. It was among the Top20 most
523 prevalent ribotypes from 2011 to 2014, and the 12th most prevalent one in 2013-2014, with a
524 prevalence of 1.9%. In France, its prevalence was very low: <1% in 2017 (Freeman et al., 2018) and
525 in 2020 (National Reference Laboratory Report, 2020; https://www.pasteur.fr/fr/sante-
526 publique/CNR/les-cnr/bacteries-anaerobies-botulisme/rapports-d-activite), and undetected in ten
527 French hospitals in 2022 (Le Monnier et al., 2022). Moreover, ribotype 017 has been described as
528 rare in animals (Imwattana et al., 2019). The high prevalence of WRT017 in the equine population
529 under study (20%) was therefore rather unexpected. It is difficult to know whether our findings are
530 indicative of an emergence of ribotype 017 in Equidae in the region or whether they fall within the
531 range of normal fluctuations of sporadic cases. In any case, the prevalence and virulence of 017 strains
532 in Equidae in Normandie is of concern.
533
534

535 **Conclusion**

536 Our study evaluated, for the first time, *C. difficile* importance in all French Equidae necropsied
537 in Normandie from 2019 to 2021, whatever the cause of animal death. It revealed a high molecular
538 diversity among *C. difficile* strains of equine origin. Thanks to a high standard diagnosis of *C. difficile*
539 infection, our study confirmed the importance of antibiotics treatment as a risk factor, and revealed the
540 virulence of WRT017 strains. Finally, Equidae in France represent a reservoir for *C. difficile* clinical
541 ribotypes which would deserve to be carefully monitored in a One Health perspective.
542

543 **Materials and Methods**

544 **Necropsies of Equidae**

545 The necropsy of dead Equidae is frequently performed to determine the cause of animal death.
546 The equine population under study comprises all Equidae necropsied at Laboratory for Animal Health
547 of ANSES in Normandy from 2019 to 2021, i.e. all examined animals without any selection except age
548 (fetuses and stillborn foals were not included). On the contrary, at the end of 2018, during preliminary
549 experiments performed to set up the procedure to identify *C. difficile*, five Equidae were specifically
550 selected on the basis of their *post-mortem* observed signs of endo-enterotoxaemia.

551 All necropsies were performed as usual in order to determine the cause of animal death, using
552 a standardized procedure (Tapprest et al., 2019). This protocol systematically includes external
553 examination of the cadaver, examination of the subcutaneous connective tissue, muscles and joints,
554 evisceration and examination of the abdominal organs, evisceration and examination of the thoracic
555 organs, isolation and examination of the upper respiratory tract, cervical esophagus, thyroid gland and
556 tongue, isolation of the head and examination of the brain, guttural pouches and nasal cavities.
557 According to animal clinical picture and observations during necropsy, complementary analyses, like
558 bacteriological and molecular analyses to identify pathogens, could also be performed.

559 For each animal, during and after necropsy as usual, several data including: individual
560 identification data (breed, sex, age, location, i.e. the premises zip code), clinical data and history,
561 including previous antibiotic treatment, observations during necropsy examination and results of
562 ordered analyses were recorded in a necropsy register. On the basis of all data, results and
563 observations as usual, the veterinarian in charge of necropsies established the cause of animal death
564 (or its suspicion), which is also registered according to the Thesaurus of equine death causes
565 developed *via* Resumeq, the French surveillance network for equine mortality
566 (https://sitesv2.anses.fr/fr/minisite/resumeq/pr%C3%A9sentation-de-resumeq), as published
567 (Tapprest et al., 2019).

568

569 **Sampling of digestive, intestinal contents**

570 During each necropsy, sampling was specifically performed for the project. Digestive contents
571 were recovered in animal caecum in all cases, and in case of an altered, watery consistency, in any
572 other intestine segment (most often the colon, but also the small intestine in a few cases). Samples
573 were aliquoted and preserved at 4°C and -20°C at the beginning of the study, and then at 4°C, -20°C
574 and -70°C.

575

576 **Usual *post*-necropsy analyses performed to identify pathogens**

577 Aliquots of intestinal samples were streaked on different selective plates. For *Escherichia coli,*
578 four colonies isolated on EMB plates (Eosin Methylene Blue) were streaked again on blood plates to
579 see hemolysis. *Salmonella* spp. could also be identified. For anaerobic species, ChromID and
580 Columbia agar + 5% sheep blood plates (BioMérieux) were used under anaerobiosis to respectively
581 identify *C. difficile* and either *C. perfringens* or *P. sordellii*. Bacteriological identification was confirmed

582 by MALDI-TOF Mass Spectrometry, using an MBT Smart mass spectrometer and MBT Compass and
583 FlexControl softwares (Bruker Daltonics). Finally, PCR could be performed to identify rotavirus.
584
585 ***C. difficile* detection and isolation specifically performed for this study**
586 *C. difficile* isolation from intestinal samples was performed as follows: an aliquot of intestinal
587 samples was directly spotted and streaked onto a commercial selective agar plate (ChromID in most
588 cases or CLO plates, BioMérieux) that was incubated 48h at 37°C under an anaerobic atmosphere.
589 In case of a negative result, an enrichment step was performed in rich broth (BHI supplemented by
590 cysteine at 0.1%, yeast extract at 5g/L, taurocholate, a primary bile salt, at 1g/L and antibiotics, D-
591 cycloserine at 250µg/ml and cefoxitin at 8µg/ml (OXOID). A digestive content aliquot was inoculated
592 in this rich medium, incubated at 37°C for 3-7 days to promote *C. difficile* spore germination and
593 vegetative cell growth while counter-selecting many enteric species, and finally plated onto ChromID
594 plates. Suspicious *C. difficile* colonies based on irregular morphology, typical smell and black
595 pigmentation on ChromID plates were confirmed by MALDI-TOF mass spectrometry.
596
597 ***C. difficile* primary characterization**
598 Our primary screening PCR was targeting *tpi*, a glycolysis house-keeping gene encoding triose
599 phosphate isomerase and specific marker of *C. difficile* species, together with *tcdA* and *tcdB* virulence
600 genes, as published (Lemee et al., 2004). In some cases, to overcome the PCR product small size in
601 the case of a truncated *tcdA* copy, another amplification was performed (Persson et al., 2011). PCR
602 products were revealed after classical gel electrophoresis.
603
604 ***C. difficile* preservation**
605 While being confirmed by both PCR and mass spectrometry, an isolated colony was grown in
606 BHI broth to preserve the strain. Preservation was not performed immediately in two cases, and the
607 corresponding strains could not be recovered again despite independent growth assays, both on
608 plates and in liquid, suggesting *C. difficile* spores to be present at a very low initial level. Isolates
609 resulting from a single black colony on ChromID were cultivated and preserved at -70°C.
610
611 **Final characterization of all *C. difficile* isolates**
612 The profile of toxin and other relevant genes (including *tcdA*, *tcdB*, *tcdC*, *cdtA* and *cdtB*) of all
613 isolates was established using a multiplex PCR followed by capillary gel electrophoresis, as previously
614 described (Le Maréchal et al., 2020). This multiplex PCR targets the following genes/loci: i) *tpi*, a *C.*
615 *difficile* gene marker, ii) PaLoc genes: *tcdA* and *tcdB* toxin genes, and *tcdC*, a negative regulator
616 showing characteristics deletions in epidemic strains, iii) PaLoc absence, as revealed by a 117 bp-
617 long *lok* fragment in non-toxigenic strains and iv) CDTLoc genes: *cdtA* and *cdtB* genes coding for the
618 binary toxin subunits.
619 Typing was also performed. The PCR-ribotype (PCR-RT) of all isolates was determined using
620 a capillary gel-based electrophoresis as described (Ferraris et al., 2019). The profiles were analyzed

621 with GeneMapper software (Thermo Fischer Scientific, Villebon-sur-Yvette, France). Strains were
622 assigned a ribotype (WRT) according to the WEBRIBO database (https://webribo.ages.at/). In
623 addition, the first 15 isolated strains were characterized by Multi Locus Sequence Typing (MLST), as
624 previously described (Griffiths et al., 2010).
625 The susceptibility of all *C. difficile* isolates to metronidazole and vancomycin was established.
626 MICs of vancomycin and metronidazole were determined by the Etest (bioMérieux) on Brucella blood
627 agar (Becton Dickinson) supplemented with vitamin K1 (10 mg/L), hemin (5 mg/L) and defibrinated
628 horse blood (5%) (Oxoid). Plates were incubated anaerobically for 24 h at 37 °C.
629
630 **Toxin detection in digestive contents**
631 *C. difficile* glutamate dehydrogenase (GDH), an antigenic biomarker of *C. difficile* species, and
632 free Toxins (TcdA and TcdB) were detected in animal digestive contents using a commercial
633 Enzymatic Immuno-Assay (Quick Check Complete, Alere), according to the supplier
634 recommendations. A total of 63 intestinal samples were tested (all 34 *C. difficile*-positive ones and 29
635 *C. difficile*-negative ones).
636
637 **Statistical analysis**
638 Association between variables was tested with Pearson's Chi-squared test and Fisher's exact
639 test where appropriate ($n < 5$). We did not impute unknown values. Differences were statistically
640 significant for a p-value <0.05.

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881

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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/enterotoxæmia										
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: Equidae n°	Isolated from: Intestinal sample	Pathogenicity: Genotype (established by PCR) *tpi* [a]	*tcdA* [b]	*tcdB* [a]	lok [c]	*tcdC* [d] deletion	*cdt* [e]	Toxin profile [f]	Type: Ribotype [g] (WRT)	MLST [h]	Antibio-resistance: 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] Iok 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 sample and the other from CDL)

[¤] animal necropsied in 2018

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

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	-	- +	
0[¤]	**CDL**	*Cd* E-0	**Toxigenic**	TcdB	017	ND	ND	ND
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

Figure 1

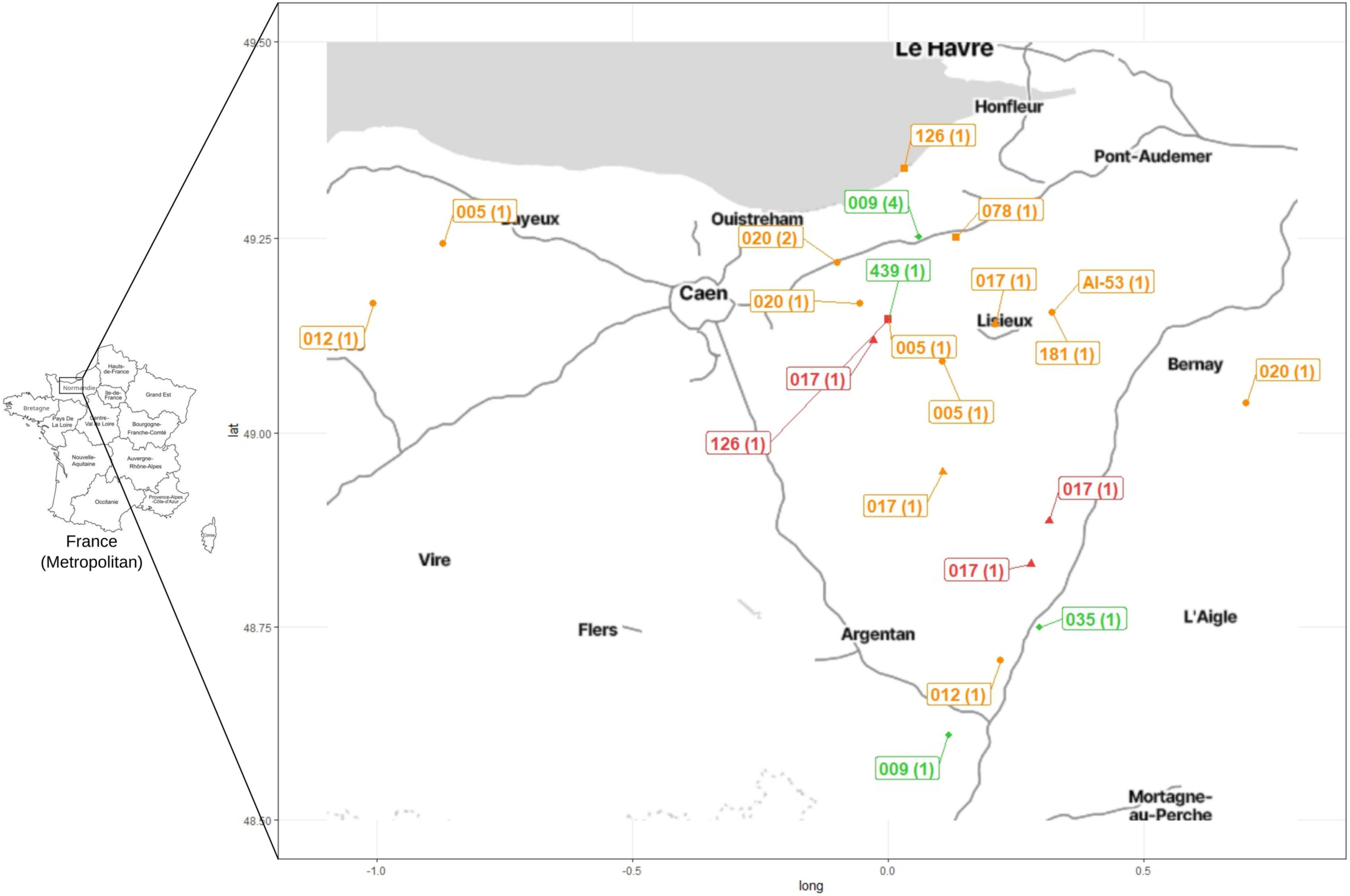
Le Havre
Honfleur
Pont-Audemer
Ouistreham
Caen
Lisieux
Bernay
Vire
Flers
Argentan
L'Aigle
Mortagne-au-Perche
126 (1)
009 (4)
078 (1)
005 (1)
020 (2)
439 (1)
017 (1)
AI-53 (1)
020 (1)
012 (1)
005 (1)
181 (1)
017 (1)
005 (1)
126 (1)
017 (1)
017 (1)
017 (1)
035 (1)
012 (1)
009 (1)
49.50
49.25
49.00
48.75
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 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 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 *C. difficile* infection. The location of animals is marked by symbols depending on the toxin profiles of the 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

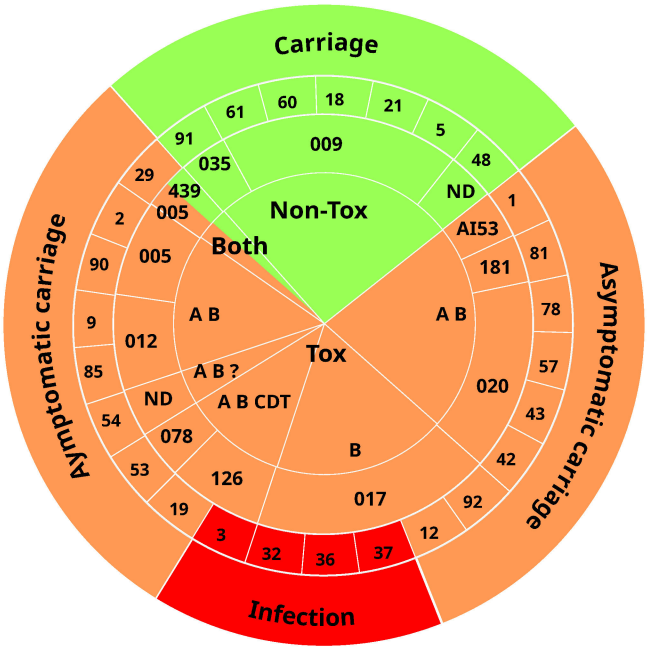
Carriage
Asymptomatic carriage
Infection
Aymptomatic carriage
Non-Tox
Both
Tox
009
035
439
005
005
012
ND
078
126
017
020
181
AI53
ND
A B
A B ?
A B CDT
B
A B
61
60
18
21
5
48
1
81
78
57
43
42
92
12
37
36
32
3
19
53
54
85
9
90
2
29
91

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 are 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 strain ability to produce CDT is unknown (the presence of *cdt* genes has not been tested by PCR.).

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

Third: Animal identification number

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