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Anaerobe 83 (2023) 102765

Contents lists available at ScienceDirect

Anaerobe

journal homepage: www.elsevier.com/locate/anaerobe

Prevalence of *Clostridioides difficile* in dogs (*Canis familiaris*) with gastrointestinal disorders in Rio de Janeiro

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ARTICLE INFO

Handling Editor: Maja Rupnik

Keywords:
Bacterial epidemiology
Clostridioides difficile
Canis familiaris
Gastrointestinal disorders

ABSTRACT

Clostridioides difficile infections (CDI) have a high morbidity and mortality rate and have always been considered a nosocomial disease. Nonetheless, the number of cases of community-acquired CDI is increasing, and new evidence suggests additional *C. difficile* reservoirs exist. Pathogenic *C. difficile* strains have been found in livestock, domestic animals, and meat, so a zoonotic transmission has been proposed.

Objective: The goal of this study was to isolate *C. difficile* strains in dogs at a veterinary clinic in Rio de Janeiro, Brazil, and characterize clinical and pathological findings associated with lower gastrointestinal tract disorders.

Methods: Fifty stool samples and biopsy fragments from dogs were obtained and cultured in the CDBA selective medium. All suggestive *C. difficile* colonies were confirmed by MALDI-TOF MS and PCR (tpi gene). Vancomycin, metronidazole, moxifloxacin, erythromycin, and rifampicin were tested for antibiotic susceptibility. Biofilm, motility assays, and a PCR for the toxins (*tcd*A, *tcd*B, and *cdt*B), as well as ribotyping, were also performed.

Results: Blood samples and colonic biopsy fragments were examined in *C. difficile* positive dogs. Ten animals (20%) tested positive for *C. difficile* by using stool samples, but not from biopsy fragments. Most *C. difficile* strains were toxigenic: six were A+B+ belonging to RT106; two were A+B+ belonging to RT014/020; and two were A-B- belonging to RT010. All strains were biofilm producers. In the motility test, 40% of strains were as motile as the positive control, CD630 (RT012). In the disc diffusion test, two strains (RT010) were resistant to erythromycin and metronidazole; and another to metronidazole (RT014/020). In terms of *C. difficile* clinicopathological correlations, no statistically significant morphological changes, such as pseudomembranous and "volcano" lesions, were observed. Regarding hematological data, dogs positive for *C. difficile* had leucopenia ($p = 0.02$) and lymphopenia ($p = 0.03$). There was a significant correlation between senility and the presence of *C. difficile* in the dogs studied ($p = 0,02$).

Conclusions: Although *C. difficile* has not been linked to canine diarrheal disorders, it appears to be more common in dogs with intestinal dysfunctions. The isolation of ribotypes frequently involved in human CDI outbreaks around the world supports the theory of *C. difficile* zoonotic transmission.

1. Introduction

Clostridioides difficile is a Gram-positive, spore-forming anaerobic bacillus that has been recognized as an important enteropathogen in both humans and animals [1,2]. In humans, it can colonize and proliferate in the gut, especially in cases of an imbalance of the intestinal microbiota, such as the use of broad-spectrum antibiotics, which leads to diarrhea and pseudomembranous colitis [3]. Most pathogenic isolates of *C. difficile* are associated with three major toxins, TcdA, TcdB, and CDT, which are the main virulence factors and promote, among other things,

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https://doi.org/10.1016/j.anaerobe.2023.102765
Received 4 April 2023; Received in revised form 18 July 2023; Accepted 19 July 2023
Available online 12 August 2023

detachment of the epithelial surface, extravasation of plasma proteins, and alteration of hydroelectrolytic transportation [4,5]. The consequence is an enteric disease known as *Clostridioides difficile* infection (CDI).

In humans, CDI was initially associated with the use of antibiotic therapy [6] in immunocompromised and hospitalized patients [3,7]. Nevertheless, the epidemiology of *C. difficile* has changed in recent decades, growing in the community environment with different risk factors in greater frequency and geographical locations not yet described [8,9]. This new pattern associated with genotypic similarity, sometimes indistinguishable, recovered from human and animal isolates suggests a zoonotic possibility [10]. The One Health concept raises concerns about transmission sources beyond the hospital environment, such as nature, food, and animals [11]. There are substantial community reservoirs and evidence of long-range interspecies transmission, probably linked to anthropomorphic factors such as intensive animal husbandry, increased travel, international trade, and indiscriminate use of antibiotics in farm animals [11]. Surveillance focused on the One Health concept of *C. difficile* from diverse human, animal, and environmental sources, and that considered the specificities of each geographic region, which is critical for developing a better understanding of the epidemiological and genetic factors that contribute to the emergence, evolution, and spread of *C. difficile* [12].

As for dogs, close contact should increase an even greater risk for transmission, especially if the pet stays indoors. Studies show different rates of isolation for *C. difficile*, ranging from 0 to 18% for diarrheal [1,2,13–15] and non-diarrheal dogs [8,16–19], reaching 58% in association with specific groups, such as age [20–22] and contact with human or veterinary health facilities [23–26]. Due to the presence of toxigenic *C. difficile* strains in asymptomatic animals and the failure to reproduce the CDI in healthy dogs with or without antibiotic treatment [27], the role of *C. difficile* in canine enteric disease remains unknown [28,29]. There have been some reports of toxigenic strains of *C. difficile* being associated with diarrhea in dogs [1,2,4,13–16,30–33], including an outbreak in a veterinary hospital [34]. It is still unknown whether *C. difficile* represents an opportunistic pathogen or is simply a fortuitous finding in this animal species.

Few reports of *C. difficile* in dogs have been published in Brazil [15,19,35,36] and none have linked the pathological findings in positive animals to diarrheal disorders. The purpose of this study was to isolate and characterize the clinical, epidemiological and pathological findings associated with *C. difficile* strains in dogs with disorders with lower gastrointestinal tract in Rio de Janeiro. *C. difficile* were also characterized according to their ribotype, antimicrobial resistance pattern, biofilm production, and motility assay.

2. Materials and methods

2.1. Animals and sampling

From 2018 to 2020, tissue, feces, and blood samples were collected from 50 domestic dogs with lower gastrointestinal tract disorders at a single diagnostic center, which works with several veterinary clinics, in the city of Rio de Janeiro. The study included all animals that underwent the colonoscopy procedure to elucidate any intestinal disease that caused diarrhea, abdominal discomfort or pain. There were no distinctions related to the age, breed, gender, diet, or clinical history of the animals. The samples were obtained at random during the colonoscopy, depending on the total number of colonoscopies performed at the diagnostic center. A fragment of a colonic biopsy fragment was collected and placed in thioglycolate broth. All tubes were kept at room temperature before being transported to the laboratory to be identified. The remaining fragments were placed in a 10% buffered formalin solution (QUIMESP QUÍMICA) and kept at room temperature for histopathological analysis. There was no standardization of the colonic biopsy site. In addition to the biopsy fragments, all animals had stool samples from de same day of the proceeds collected in a sterile screw-top container. Many of the fecal samples were obtained with the aid of an enema and the rest spontaneously. The vials were kept frozen at −20 °C, until they were processed for *C. difficile* identification and isolation. Blood was also collected from the animals for hematological and biochemical analyses. All animal procedures in this study were approved by the institutional ethics committee of Universidade Federal do Rio de Janeiro-Rio de Janeiro (CEUA-UFRJ; protocol number 164/19).

2.2. Epidemiological data

A clinical epidemiological survey was completed by the tutors of the animals under study. This study looked at basic epidemiological data, such as age, race, and residential neighborhood; characteristics of the gastrointestinal disorder (aspect, frequency, duration, and treatment); whether the animals used antibiotics; and their clinical history, as well as some habits of the animal and its tutors. Animals up to 6 months old were classified as puppies; those from 6 months to 7 years old as adults; and those over 7 years old as seniors or geriatrics.

2.3. Endoscopic scoring

The images obtained during the colonoscopy procedure were categorized based on BSAVA (British Small Animal Veterinary Association, 2008) criteria for tone, integrity, vascularization, consistency, intestinal caliber and content, and the presence of hemorrhage or other lesions. All analyses were conducted blindly and performed by two different pathologists.

2.4. Histopathological classification of colonic lesions

The tissue samples were all fixed in a 10% neutral buffered formalin solution and embedded in paraffin wax after being trimmed. Paraffin-embedded tissues were sectioned at a thickness of 5 μm and stained with hematoxylin and eosin (HE). The histopathological classification was performed by three different pathologists for the histopathological examination. Damage to the lining epithelium, alterations of Lieberkuhn's Crypts (epithelial injury, dilation, and/or distortion), the concentration of goblet cells (hypoplasia or hyperplasia), circulatory alterations (hyperemia, edema, or hemorrhage), fibrosis, necrosis, superficial deposition, and/or nature of the inflammatory cells were all examined (neutrophilic, eosinophilic, lymphoplasmacytic, or histocytic) [37–39].

2.5. Hematological and biochemical analyses

Hematological analyses were performed manually, using a Neubauer chamber, microhematocrit centrifuge, and microscopic examination of the blood smears. The packed cell volume was determined using a microhematocrit centrifuge, and the reading was performed on an appropriate card from the same manufacturer. In the Turk solution, the total number of leukocytes (WBC) was analyzed in the Neubauer chamber. Using standard formulas and microscopic correlation, the mean corpuscular volume (MCV) and mean corpuscular hemoglobin concentration (MCHC) were calculated. Differential leukocyte counts were performed on 100 cells from blood smears stained by rapid panotic and examined under a light microscope. Platelets were counted indirectly using an average count of ten fields in the terminal region of the blood smear under a light microscope (*Bioval – L 1000B*).

The concentrations of albumin, alkaline phosphatase, alanine aminotransferase (ALT), gamma-glutamyl transferase (GGT), urea, creatinine, cholesterol and triglycerides were photometrically determined using a semi-automatic biochemical analyzer (Bioplus – BIO 200) and analytical kits (Labtest Diagnóstica).

2.6. Isolation and identification of Clostridioides difficile

The rapid C. diff Quik Chek Complete (Alere®) enzyme immunoassay, which detects glutamate dehydrogenase – GDH antigen and toxins, was used to screen all fecal samples. The test was performed according to the manufacturer's recommendations. Regardless of the results of the enzyme immunoassay, all samples were cultured in *Clostridioides difficile* Brucella Agar (CDBA) a selective culture medium, with sodium taurocholate (0.01%), D-cycloserine (500 mg/L) and cefoxitin (0.0128 mg/L).

Before inoculation in the culture medium, the fecal specimens were subjected a heat shock treatment for 1h at 60 °C to reduce the intestinal microbiota [40]. Following this incubation, approximately 1.0 g of feces was solubilized in conical tubes containing 1 mL of buffered saline and vigorously vortexed until the contents became homogeneous. Following the shock, 100 μL of each sample was seeded on CDBA plates. The plates were incubated for at least 7 days at 37 °C under anaerobic conditions (80% of N2, 10% of H2 and 10% of CO2) in a Glove Box (Coy Labs). The biopsy samples, which were placed in thioglycolate broth, were inoculated in Blood Agar and CDBA, with no heating shock. All plates were incubated in anaerobic conditions, under the same conditions previously mentioned, for 48h into Blood Agar and 7 days into CDBA. Following this period, all colonies of both media were observed for the colonial appearance using a stereoscopic microscope (Carl-Zeiss, Jena). Gram staining was performed on colonies with typical morphology, the appearance of broken glass, and the odor of "horse manure". To confirm species identification, all gram-positive bacilli colonies were subjected to mass spectrometry (MALDI-TOF/MS; Bruker®). The sample for analysis by MALDI-TOF MS was prepared by placing the colonies in the target plate and coating them with 1 μL of 70% formic acid, letting it dry at room temperature (TA); and coat them with 1 μL a 10 mg/mL of α-Cyano-4-hydroxycinnamic acid (70/30 acetonitrile/water with 0.1% trifluoracetic acid -TFA). After drying at RT in the dark the analysis was made. After MALDI-TOF MS confirmation (scores ≥2,3), *C. difficile* strains were genotypically confirmed by PCR in the strains positive for the species-specific gene, triose phosphate isomerase (*tpi*) [84].

2.7. Antibiotic susceptibility test

Disk diffusion was performed as described by Fraga et al. (2016). The antimicrobials tested were 5 μg metronidazole (MTZ), 5 μg moxifloxacin (MXF), 5 μg erythromycin (ERY), 5 μg rifampicin (RIF) and 5 μg vancomycin (VAN). Strains were grown on Brucella agar plates supplemented with hemin (5 μg/mL) and vitamin K (1 μg/mL) for 24 h at 37 °C, under anaerobic conditions (80% of N_2, 10% of H_2 and 10% of CO_2) in a glove box. Before the test, an inoculum corresponding to the tube 1.0 of the McFarland scale (~3.0×10^8 CFU/mL) was prepared. For the inoculum on the plates, a sterile cotton swab was dipped in the suspension and spread evenly across the plates. The antibiotic discs (Oxoid, Basingstoke) were placed on the plates and after incubation for 24h under anaerobic conditions. The next day, the zone diameters (mm) within the area of inhibited bacterial growth were measured. Strains susceptible to the antibiotics according to the following measurements: VAN ≥19 mm; MTZ ≥23 mm; MXF ≥20 mm; ERY ≥20 mm; RIF ≥30 mm. The *C. difficile* R20291 strain (027 ribotype) was used as a control [41,42].

2.8. Motility test

As described by Chunhui Li *et* al. [43], for motility assay an agar medium was prepared (37 g/L BHI broth medium, 0.3% agar [w/v] BD Biosciences Brazil®) and 25 mL poured into Petri dishes. The soft agar plates were then air-dried for 10 min in a laminar flow hood with airflow, transferred into the anaerobic chamber, and allowed to reduce inside the anaerobic chamber overnight before inoculation with *C. difficile* strains. A single colony of *C. difficile* was inoculated in the center of the plate test, which was then incubated for 72 h at 37 °C under anaerobic conditions (80% of N_2, 10% of H_2, and 10% of CO_2). The swimming motility of the isolate was quantitatively determined by measuring the radius (millimeters) of the zone of motility at three different time points (24, 48, and 72 h). All motility assays were performed in triplicate (three distinct plates). The strain CD630 (ribotype 012) was used as a positive control.

2.9. Biofilm production assay

The biofilm production assay was performed as recommended by Pantaléon et al. [44] in 24-well polystyrene plates. Briefly, an overnight culture of *C. difficile* strains was diluted (1:100) into a fresh BHI-PRAS containing 1.8% glucose (Sigma), and 1 mL of this culture was distributed in each well. All tests were made in triplicate and the CD630 (RT012) *C. difficile* strain was used as a positive control. To avoid evaporation 1 mL of sterile 1x PBS (0.1M) was added to empty wells. The plate was incubated under anaerobic conditions at 37 °C for 3 days. After incubation, the media was removed out of the wells and gently washed with 1x PBS (0.1M). The plate was incubated for 10 min at 37 °C for drying, then fixed (90% ethanol and 5% acetic acid) for 20 min at RT. After removing the fixing solution, the plate dried for 10–20 min at 37 °C, and crystal violet (0.2% w/v) was added in each well and incubated at 37 °C for 30 min. The wells were washed 2x with 1 x PBS and the absorbance value measured in a spectrophotometer at 570 nm (Ultrospec 2000 UV/Visible Spectrophotometer®, Pharmacia Biotech). As a negative control, BHI-PRAS media was used for background staining quantitation, and the value obtained was subtracted from each sample measurement. Strains were considered biofilm producers as follows: OD ≤ ODc (no biofilm); ODc < OD ≤ 2xODc (weak); 2xODc < OD < 4xODc (moderate); OD > 4xODc (strong); 3x ODc = cut off value average of the OD of the negative control; OD = average OD of a strain minus ODc.

2.10. Molecular characterization of C. difficile

To obtain the bacterial genomic DNA, *C. difficile* isolates were grown anaerobically in Blood Agar plates for 18 h at 37 °C. Three to five colonies were added to a solution containing 5% of Chelex 100 (Bio-Rad) and 2% of Proteinase K (20 mg/mL; Sigma-Aldrich) and the DNA extractions were obtained according to the manufacturer's instructions. DNA samples were kept at −20 °C until use.

The presence of toxin A (*tcd*A), toxin B (*tcd*B) and binary (*cdt*B) genes was evaluated by a protocol described by Wroblewski et al. [45,47]. The final volume of each reaction for the detection of toxin genes was 25 μL each, which included 12.5 μL of Master Mix (Promega), 1.25 μL of primers (0.5 pmol/μL), and 3 μL of DNA in sterile water. All amplification reactions were performed in a thermocycler (Applied Biosystems Veriti 96 – well ThermalCycler) and the cycling run conditions being 95 °C for 5 min; followed by 30 cycles of three steps of 94 °C for 30 s, 50 °C for 30 s and 72 °C for 40 s; and finishing with a final extension at 72 °C for 5 min. Five microliters of the PCR reaction were mixed with the Blue Green Loading dye I (LGC) and the electrophoreses was made in 1x TAE running buffer (0.1 M Tris, 1M acetic acid and 0.5 M EDTA) on a 1.0% agarose gel (Sigma-Aldrich) for 1h at 100 V. Following the run, the gel was observed under UV transillumination (MiniBis Pro® Dnr Bio-Imaging System), and the sizes of the amplicons compared to the 1 Kb ladder (Invitrogen). In all reactions, *C. difficile* strain R20291 (RT027) was used as a positive control.

The isolates were also characterized by Bidet et al. [46] using an agarose gel-based PCR-ribotyping method, which amplifies the intergenic regions of the 16S–23S ribosomal subunit. However, the primer sequence used was similar to that used by Aldape et al. [47]. The PCR mix consisted of 5 μL of Green GoTaq® G2 5x buffer (Promega), 0.5 μL dNTP (0.2 mM), 1.25 μL of each primer (0.5 pmol/μL), 0.2 μL of Taq polymerase (5U/μL), 5 μL of DNA and sterile water until a final volume of 25 μL was reached. The following cycle was used to perform the

amplification reaction: initial denaturation of 94 °C for 3 min; 34 cycles of 94 °C for 1 min, 57 °C for 1 min and 72 °C for 1 min; and a final extension of 72 °C for 5 min, followed by ending at 4 °C. After amplification, PCR products were submitted to an electrophoresis in 2% agarose gel (SeaKem® Gold Lonza) in 1x TBE (89 mM Tris, 89 mM boric acid and 2 mM EDTA) buffer at 85 V, 400 mA for 3h. The gel was submerged in a 0.5 μg/mL ethidium bromide solution for 30 min to visualize the amplicons. The banding patterns were analyzed using BioNumerics® software (Applied Maths®, Belgium) in comparison to the 100 bp DNA ladder (Invitrogen). The nomenclature of the ribotypes found was designated following the Cardiff/Leeds standard (001, 002, 014, etc).

3. Results

C. difficile was found in ten of the 50 dogs studied, accounting for 20% of dogs with disorders in the lower gastrointestinal tract. In this study, animal fecal material was used to isolate the bacteria because the colonic biopsy fragments were negative. Other *Clostridium* genus members were co-isolated and identified by MALDI-TOF MS, such as *C. perfringens* and *C. paraputrificum*, but *C. difficile* was positive in 40% (4/10) samples. *C. difficile* isolates from eight of ten (80%) were toxigenic and belonged to ribotypes 106 (6/10) and 014/020 (2/10). The remaining two isolates (20%) were non-toxigenic and belonged to ribotype 010. The C. diff Quik Chek Complete test detected only 12.5% (1/8) of the toxigenic samples. The binary toxin CDT was not found in any isolate.

Clinical and epidemiological parameters for our study were examined in general, based on all negative and positive samples and by ribotype. In this group of animals, advanced age was found to be a significant ($p < 0.05$) and relevant risk factor, with 90% (9/10) of positive dogs for *C. difficile* being 7 years old or older. Although it had the greatest age diversity, with a single 2-year-old specimen, RT106 maintained an average age of 9.1 years among the animals where it was isolated. Because all *C. difficile*-positive animals had clinically manifested diarrhea, this was not deemed a significant factor ($p = 0.24$). The frequency with which the animals visited veterinary institutions ($p = 0.15$) and pet stores ($p = 0.15$) had no effect on the occurrence of *C. difficile* colonization. The same was found for concomitant diseases ($p = 1.00$) and the other parameters investigated: antibiotic therapy, corticosteroid therapy, hospitalization, and contact between the animal's tutor and hospital environments for professional or even health reasons. The clinical and epidemiological parameters' ribotype analyses were not significant. Table 1 contains the main information about the dogs who tested positive for *C. difficile*.

Fig. 1 summarizes the antimicrobial susceptibility pattern found in this study. Resistance was observed in three strains (ES14, ES20, and ES48) from the positive dogs for *C. difficile*. ES14 (RT010) and ES48 (RT014/020) showed resistance to MTZ and reduced susceptibility to VAN, whereas ES20 (RT010) was only resistant to MTZ. For the other microbials tested, there was variable resistance regarding ERY and MXF. No strain showed resistance to RIF.

The biofilm assay revealed that all *C. difficile* strains were biofilm producers, but isolates ES14 (RT010), ES32 (RT106), and ES39 (RT106) were strong biofilm producers (Fig. 2). Conversely, the motility test revealed that ES11 (RT106), ES14 (RT010), ES16 (RT106), and ES20 (RT010) were as motile as the positive control, CD630 (RT012) (Fig. 3).

Considering the parameters that most change in human CDI, the hematological and serological data of greater relevance to our work were grouped according to all the animals in the study (Table 2) and according to the ribotypes of the *C. difficile* positive samples (Table 3). Global leukometry revealed changes in 50% of the positive animals, with 40% falling below the species' normal values. Only leukopenia was statistically significant among *C. difficile* positive animals ($p = 0.02$). Regarding the results of the specific leukometry, 80% of the positive animals for C. difficile had lymphocytes below the normal limit in the absolute values – lymphopenia ($p = 0.03$) – and 40% had absolute neutrophils above the species' normal parameters for – neutrophilia ($p = 0.70$). The ratio of neutrophil and lymphocyte counts (NLR) is currently used as an important prognostic parameter in *C. difficile* infections. The NLR of 40% of the positive animals was greater than 10, indicating a more unfavorable prognosis in these animals. Despite the lack of statistical significance, both RT010 strains (100%) had an unfavorable NLR.

Pathologically, no macro or microscopic changes associated with *C. difficile* infection, like pseudomembranes or volcano lesions, were observed in the dogs in this study. Histopathological examination of colonic biopsy fragments revealed morphological alterations common to different etiologies, such as epithelial injury, distortion in Lieberkuhn's crypts, and modification in the concentration of goblet cells, in addition to the presence of an inflammatory infiltrate, edema, hemorrhage, necrosis, and a surface deposit (Fig. 4). While 90% of the positive samples had epithelial injury, edema, and hyperemia, there was no statistical significance in any of the parameters analyzed (Table 4), nor in the comparison by ribotype.

4. Discussion

The current study included only animals with diarrheal disorders and found that 20% of the animals tested positive for *C. difficile*, corroborating the higher rates of isolation [35,48,49]. However, the prevalence did not appear to be associated with any of the groups, such as puppies [21,22,50], hospitalized animals [51], those who visited veterinary hospitals [23,24,26], and those who attended human health facilities [25,52]. Comparing with the study conducted by Rainha et al. [15] with asymptomatic dogs from the same geographic region, in our study the

Table 1
Main data from dogs that tested positive for *Clostridioides difficile* and molecular characteristics of the isolates.

Dogs	Age (≥7 years)	Presence of diarrhea	Previous antibiotic treatment	Hospital contactants	Previous hospitalization	Co-isolation with other potential agents	Toxin profile	[a]Ribotype profile	[b]Alere test
ES11	+	+	–	–	–	–	$A^+B^+CDT^-$	106	+
ES14	+	+	+	–	+	+ (*Clostridium perfringens*)	$A^-B^-CDT^-$	010	+
ES16	+	+	+	–	–	+ (*Clostridium perfringens*)	$A^+B^+CDT^-$	106	+
ES20	+	+	+	–	+	+ (*Clostridium perfringens*)	$A^-B^-CDT^-$	010	+
ES32	+	+	–	+	–	–	$A^+B^+CDT^-$	106	+
ES33	–	+	+	–	–	+ (*Clostridium paraputrificum*)	$A^+B^+CDT^-$	106	+
ES39	+	+	+	–	+	–	$A^+B^+CDT^-$	106	+
ES43	+	+	–	–	+	–	$A^+B^+CDT^-$	014/020	+
ES44	+	+	–	–	–	–	$A^+B^+CDT^-$	106	+
ES48	+	+	–	–	–	–	$A^+B^+CDT^-$	014/020	+

[a] *Clostridioides difficile* ribotypes according to the molecular biology methodology ribotyping-PCR. A^+ - positive for the gene *tcd*A (toxin A); B- positive for the gene *tcd*B (toxin B); CDT^- Negative for the binary toxin gene (*cdt*B); (+): yes; (−): no.
[b] - C diff Chek complete (Alere®): (+): indicates positivity for GDH (*C. difficile*).

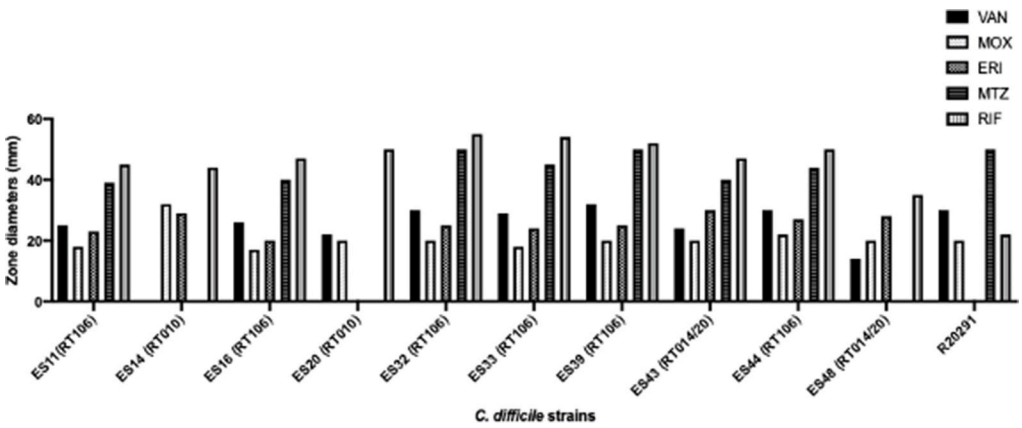

Fig. 1. Antimicrobial susceptibility test of *Clostridioides difficile* strains isolated from dogs of this study. The antimicrobial agents tested included vancomycin (VAN), moxifloxacin (MOX), erythromycin (ERI), metronidazole (MTZ) and rifampicin (RIF). Resistance was observed in three strains (ES14, ES20 and ES48). ES14 (RT010) and ES48 (RT014/020) showed resistance to MTZ and VAN, whereas ES20 (RT010) was only resistant to MTZ. The R20291 (RT027) was used as a positive control in the disk diffusion test.

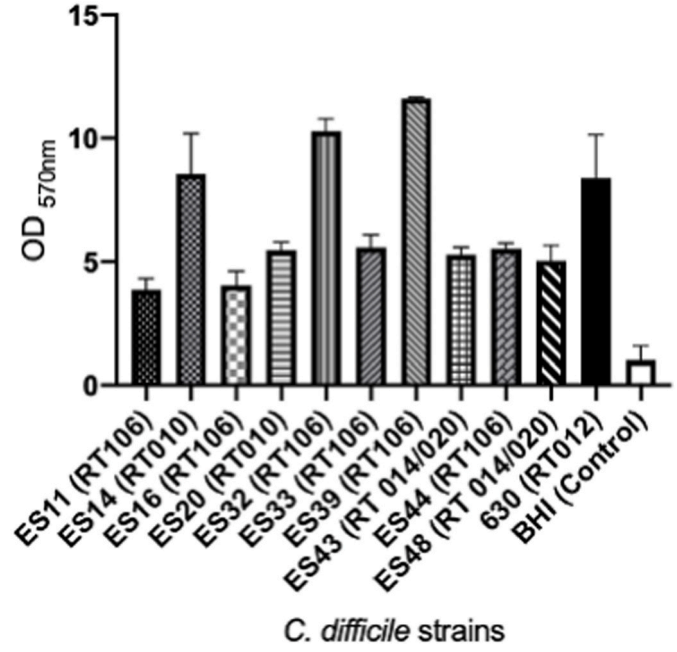

Fig. 2. Biofilm production assay of the *Clostridioides difficile* strains isolated in this study from dogs with colitis. The strains ES14, ES32 and ES39 were strong biofilm producers; ES11, ES16, ES20, ES33, ES43, ES44 and ES48 were moderate biofilm producers. The CD630 (RT012) and BH-PRAS were used as positive and negative controls. Measurements were taken based on two different biological assays with strais in triplicate.

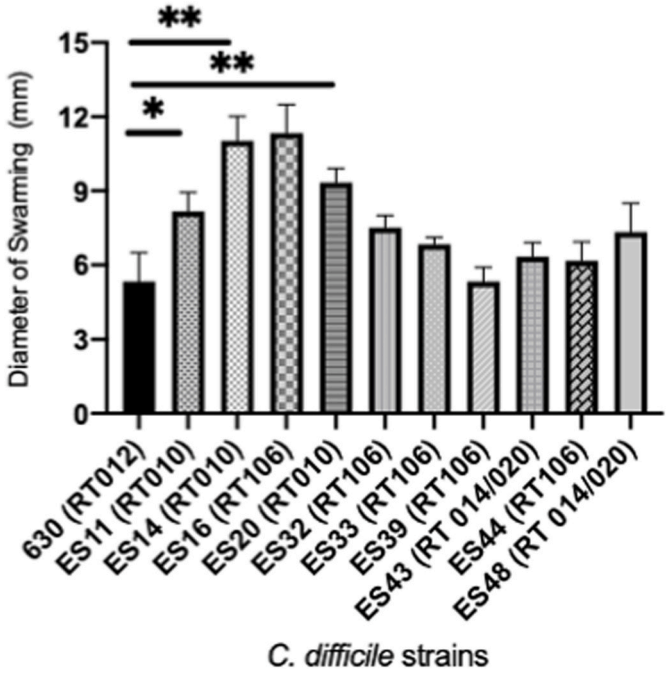

Fig. 3. Swimming motility assay of the *Clostridioides difficile* strains isolated in this study. The strains ES11, ES14, ES16 and ES20 were considered as motile as the positive controle, CD630 (RT012). Measurements were taken based on three different biological assays with strais in duplicate.

prevalence rate of *C. difficile* isolation was higher.

We attempted unsuccessfully to isolate *C. difficile* from endoscopy biopsy fragments to provide a sampling alternative for its detection, possibly due to the small sample size associated with the use of washing solution for better colonoscopy visualization. Tang et al. [53] had already reported such difficulty in microbiota studies, justifying the result by the marked decrease of the microbiota in quantity and variety caused by washing solutions, the irregular distribution of microorganisms in the lumen, and the small surface area. In this manner, the fecal sample was used to isolate *C. difficile*. *C. difficile* was identified using two diagnostic methods, as recommended by ESCMID. Even though the ALERE® C. diff quik chek Complete kit was only used for human samples, it proved to be an excellent screening test, detecting GDH in 100% of positive samples. It should be noted that our findings were comparable to those found in humans by other authors using the rapid kit, who reported sensitivity, specificity, and negative predictive value greater than 93% for the species [54–56]. On the other hand, the same kit's search for toxins performed worse than previously reported [56,57]; however, we were able to identify the genes responsible for the toxins. To confirm it and obtain more diagnostic information, we performed the cultivation, followed by colony isolation and species confirmation using mass spectrometry (MALDI-TOF MS) and PCR (toxins and ribotyping).

Antibiotic therapy, corticosteroid therapy, and hospitalization were not found to be risk factors for canine nosocomial colonization in our study. This finding supports what was previously reported by other authors [23,48,58,59], which points to a community origin observed by Ref. [10]. It is worth remembering that one of the issues with the community profile is a lack of knowledge about the risk factors associated with such a profile.

In terms of the animal's age, there appears to be a high colonization rate associated with their first weeks of life [20,21,50]; however, this attribute due to the absence of neonates in the study, could not be analyzed. The decline in gastrointestinal microbial diversity with advancing age has already been demonstrated in several animal species [60], resulting in a progressive decline in immune function, making the

Table 2
Hematological and serological data of the dogs included in the study.

Hematological and Serological	Prevalence in animals negative for *C. difficile*	Prevalence in animals positive for *C. difficile*	*P* Value
	%	%	
Low packed cell volume/ Anemia	36 (14/38)	50 (5/10)	0.48
High Global Leukometry/ Leukocytosis	15 (6/38)	10 (1/10)	1.00
Low global leukometry/ Leukopenia	8 (3/38)	40 (4/10)	0.02
Absolute value of low lymphocytes/ Lymphopenia	40 (15/37)	80 (8/10)	0.03
Neutrophil/lymphocyte (greater than 10)	24 (6/37)	40 (4/10)	0.42
Low albumin	24 (9/38)	20 (2/10)	1.00

Table 3
Hematological and serological data of the dogs included in the study positive for *Clostridioides difficile* according to the ribotype.

Clinical-epidemiological data	RT 106		RT 010		RT 014/020	
	%	*p* Value	%	*p* Value	%	*p* Value
Low packed cell volume/ Anemia	50	1.00	100	0.44	0	0.44
High Global Leukometry/ Leukocytosis	83	0.1	0	1.0	0	1.0
Low Global Leukometry/ Leukopenia	50	0.19	0	0.46	50	1.0
Absolute value of low lymphocytes/Lymphopenia	66	0.46	100	1.00	100	1.00
Neutrophil/lymphocyte (greater than 10)	33	0.19	100	0.13	0	0.46
Low albumin	0	0.13	100	0.02	0	1.00

Statistical significance of p value ($p < 0.05$); RT- Ribotype.

individual susceptible to infections and autoimmune and neoplastic diseases [61]. In humans, senility is one of the major risk factors for CDI [62]. In our study, older, senior, or geriatric animals had a higher prevalence of *C. difficile* (p = 0.02), as reported by Refs. [14,59]. Nonetheless, this characteristic does not appear to be limited to the community profile, as reported by Struble et al. [4] in a similar pattern in a teaching hospital.

When compared to other studies involving diarrheal dogs, a much higher number of toxigenic isolates of *C. difficile* (80%) were recovered [10,15,51,63]. They were all positive for toxins A and B, but negative for the binary toxin, CDT. The *C. difficile* isolates in this study belong to RT106 (6/10), RT014/020 (2/10), and RT010 (2/10). RT106 was the most prevalent in our study, which was not surprising given that this is the most isolated ribotype in dogs. Some studies suggest that RT106 rapid spread occurred at the expense of the strain's high resistance to environmental decontamination [64,65] and a variety of potential animal reservoirs, including dogs [66], coatis [36], and mollusks [67]. This ribotype has also been linked to human-acquired CDI (HA-CDI) [68,69] and CA-CDI [70,71]. The RT014/020 is also widely distributed geographically [72–74] and isolated in several animal species [36,66,75], including domestic dogs [49,63,76]. In addition to reports of CDI in hospitalized patients in Brazil [72,77], the isolation of this ribotype in various species suggests that humans and animals have a high adaptative capacity [66]. Concerning RT010, this ribotype has been identified as one of the most frequently isolated strains from humans and dogs in Europe [31,49,78], and is commonly associated with healthy people, but it has also been found in dogs with digestive disorders [18].

Two strains belonging to ribotypes, RT014/020 and RT010 were resistant to metronidazole and showed reduced susceptibility to vancomycin, respectively. Antibiotic resistance in *C. difficile* isolates from humans and dogs is becoming more common [2,18,63] and both antimicrobials have long been used as first-line drugs for the treatment of CDI [79]. Metronidazole is widely used for treating several diseases in dogs, especially diarrhea, and we have not identified strains resistant to this antimicrobial in dogs in Rio de Janeiro previously. Due to its widespread use in the treatment of Giardia infections, recent studies have suggested a phenotype of reduced susceptibility to MTZ in dogs [32,59,63]. This potential risk for the emergence of *C. difficile* strain resistance to the current first-line antibiotics associated with the ability of its pathogenicity locus (PaLoc) transfer from toxinogenic to non-toxinogenic strains, as described by Brouwer et al. [80], raised significant concerns in public health and in the therapeutic protocols in the veterinary medical clinic. Conversely, vancomycin is not an approved drug to be used in dogs in Brazil, and still, non-toxigenic strain presented a reduced susceptibility profile to this drug. Some strains isolated in our study were also resistant to erythromycin and moxifloxacin.

There have been no reports of clinical laboratory changes in dogs, caused by the presence of *C. difficile*. In contrast to what is typically seen in human patients with CDI, 40% of the dogs in our study had leukopenia (p = 0.02). Although infectious processes cause leukopenia that progresses to sepsis [81], more information about the clinical history of the animals is needed to rule out other etiologies, such as hemoparasitosis, viruses, neoplasms, autoimmune diseases, drugs, and so on. Lymphopenia was found in 80% of *C. difficile*-infected dogs (p = 0,03), correlating with human medicine in cases of recurrence and implying that this parameter can also be used to aid in the diagnosis of CDI in veterinary medicine. Recently, the ratio of neutrophil and lymphocyte counts (NLR) has been associated with a variety of inflammatory conditions, including *C. difficile* infection [82] as a prognostic factor. The NLR was greater than ten in 40% (4/10) of the positive animals, indicating a poor prognosis. Even though there was no statistical significance, two of the dogs with an NLR greater than ten belonged to RT010. When the animals from which RT010 strains were isolated were compared individually, they still showed hypoalbuminemia ($p < 0.05$) and a more accentuated pattern of anemia, as well as a more severe clinical presentation with marked hematuria and progression to death.

The colonoscopy characteristics of this study were obtained from a variety of third-party professionals, making the macroscopic analysis impossible to correlate due to a lack of standardization. The most common changes, in any case, were hyperemia, edema, hemorrhage, erosions, ulcerations, and increased mucoid content. No pseudomembranous were found in any of the animals studied. Because all the animals were symptomatic, the presence of colitis was not a positive predictor of *C. difficile strains*. Even though some of the microscopic changes associated with CDI were frequently observed, no statistically significant changes were found. Half of the *C. difficile* positive samples had varying degrees and distributions of neutrophilic infiltrates, but none of them had typical "volcano" lesions.

C. perfringens was isolated from three positive samples, one positive sample with *C. difficile* RT106 and two with RT010. Some research groups have reported the co-isolation of *C. perfringens* and the previous association of these species with diarrheal disorders in dogs [10,35,83], but future research must rule out the possibility that these species proliferated secondary to a dysbiosis of unknown etiology.

Some studies have reported a link between diarrheal disorders in dogs and the presence of toxigenic strains of *C. difficile* in feces [35,51,58]. However, the prevalence rates of toxigenic *C. difficile* found in apparently healthy animals [28,29] make it difficult to determine whether the disease is subclinical in these animals or if they are only carriers. Weese et al. [10] contended that, despite being asymptomatic carriers, dogs can become symptomatic carriers when exposed to risk factors. There is still debate over whether *C. difficile* is a primary or secondary pathogne [16]. All the dogs in our study had some sort of

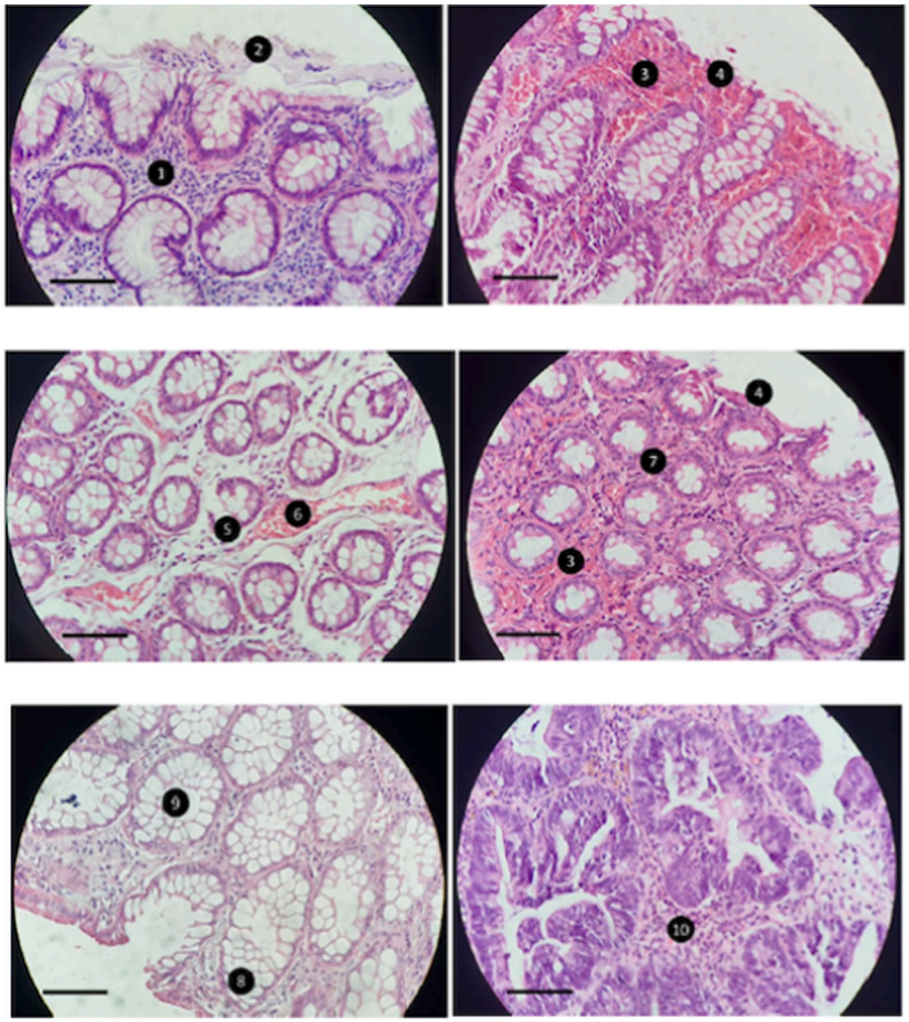

Fig. 4. Cross-sectional histological sections of the colonic mucosa of animals positive for *Clostridioides difficile* in this study stained with hematoxylin-eosin and observed under light microscopy. Numbers indicate the histopathological alterations: 1- Lymphoplasmacytic inflammatory infiltrate in the lamina propria; 2- Surface deposit; 3- Hemorrhage; 4- Lining epithelium injury; 5- Edema; 6- Hyperemia; 7-Goblet cell hypoplasia; 8- Goblet cell hyperplasia; 9- Dilation of Lieberkuhn's Crypts; 10- Pleocellular inflammatory infiltrate with predominance of neutrophils in the lamina propria. Each scale corresponds to 500 p.m.

Table 4
Histopathological changes associated with colonic biopsy fragments from the dogs included in this study.

	Prevalence in animals negative for *C. difficile*	Prevalence in animals positive for *C. difficile*	*p* Value
	%	%	
Injury to the lining epithelium	92 (37/40)	90 (9/10)	1.00
Cryptal epithelium injury	77 (31/40)	90 (9/10)	0.66
Dilation of Lieberkuhn's Crypts	80 (32/40)	80 (8/10)	1.00
Lieberkuhn's Crypt Distortion	25 (10/40)	10 (1/10)	0.42
Goblet cell hypoplasia	45 (18/40)	30 (3/10)	0.48
Goblet cell hyperplasia	22 (9/40)	30 (3/10)	1.00
Edema	55 (22/40)	90 (9/10)	0.06
Bleeding	50 (20/40)	50 (5/10)	1.00
Hyperemia	57 (23/40)	90 (9/10)	0.07
Necrosis	45 (18/40)	50 (5/10)	1.00
Lymphoplasmocytic inflammation	37 (15/40)	40 (4/10)	1.00
Neutrophilic inflammation	17 (7/40)	50 (5/10)	0.10
Surface deposit	50 (20/40)	60 (6/10)	0.72

Statistical significance of p value ($p < 0.05$).

diarrheal disorder. Despite the suspicion of *C. difficile* pathogenic potential, it was not possible to establish statistical significance between the presence of this species and diarrhea. In this regard, we believe that further studies on the microbiota of colonized animals will be required to investigate species-specific interactions that aid in the understanding of the pathogenic process in these animals.

Regardless of pathogenicity, the presence of genetically indistinguishable strains recovered from dogs and humans in common geographic regions [22] highlights the importance of epidemiological monitoring and the evolution of research on the subject. Despite the lack of evidence of direct transmission of *C. difficile*, the current study isolated ribotypes known to be responsible for human CDI outbreaks from dogs, raising the possibility of zoonotic disease. Furthermore, identifying *C. difficile* strains associated with the environment, food, and animals is critical for the implementation of preventive measures and developing strategies to improve food safety and protect human and animal health.

5. Conclusions

Several studies from around the world suggest household pets as carriers and potential sources for pathogenic *C. difficile* to humans. This study provides a better understanding of the prevalence, diversity and resistance pattern of *C. difficile* colonization in dogs over time and space, implying some degree of interspecies movement or common source of exposure. Our study isolated 20% of *C. difficile* from diarrheic dogs in Brazil, all of which belonged to ribotypes commonly found in humans -

106, 014/020 and 010 - and were mostly toxigenic. Resistance to metronidazole, moxifloxacin and erythromycin was found in strains isolated in our study. Future research will look into the clinicopathological aspects of diarrheic and non-diarrheic animals to clarify the pathogenic course in dogs. In addition to an increase in the frequency of CDI and the emergence of increased antimicrobial resistance. *C. difficile* surveillance from various human, animal and environmental sources is critical for developing a better understanding of the epidemiological and genetic factors that contribute to the emergence, evolution and spread of *C. difficile*.

CRediT authorship contribution statement

Suzana Leite: designed the project outline, conducted the literature research, provided biological samples, performed hematological, serological and histopathological analysis, wrote the manuscript. **Carlos Cotias:** provided biological samples. **Kelly C. Rainha:** performed bacterial culture and molecular analysis. **Wiep Klaas Smits:** performed the PCR-ribotyping. **Céline Harmanus:** performed the PCR-ribotyping. **Mayara Gil Santos:** performed bacterial culture and molecular analysis. **Bruno Penna:** reviewed and edited the manuscript. **Renata F. F. Moraes:** conceptualized the idea, designed the project outline. **Eliane de Oliveira Ferreira:** conceptualized the idea, designed the project outline, performed bacterial culture and molecular analysis, reviewed and edited the manuscript. All authors contributed to the article and approved the submitted version.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

No data was used for the research described in the article.

Acknowledgments

The authors gratefully acknowledge the Brazilian support agencies Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Fundação de Amparo à Pesquisa do Estado do Rio de Janeiro (FAPERJ) grant # E-26/211.554/2019 (Programa Rede de Pesquisa em Saúde) and Coordenação de Aperfeiçoamento de Pessoal de Nível Superior - Brasil (CAPES) - Finance Code 001 for supporting this study.

References

[1] J. Czepiel, M. Dróżdż, H. Pituch, E.J. Kuijper, W. Perucki, A. Mielimonka, S. Goldman, D. Wultańska, A. Garlicki, G. Biesiada, *Clostridium difficile* infection, Eur. J. Clin. Microbiol. Infect. Dis. 38 (7) (2019) 1211–1221, https://doi.org/10.1007/s10096-019-03539-6.

[2] S.C. Lim, D.R. Knight, T.V. Riley, Clostridium difficile and one health, Clin. Microbiol. Infection 26 (7) (2020) 857–863, https://doi.org/10.1016/j.cmi.2019.10.023.

[3] C.P. Kelly, C. Pothoulakis, J.T. LaMont, *Clostridium difficile* colitis, N. Engl. J. Med. 330 (4) (1994) 257–262, https://doi.org/10.1056/NEJM199401273300406.

[4] A.L. Struble, Y.J. Tang, P.H. Kass, P.H. Gumerlock, B.R. Madewell, J. Silva Jr., Fecal shedding of *Clostridium difficile* in dogs: a period prevalence survey in a veterinary medical teaching hospital, J. Vet. Diagn. Invest. 6 (3) (1994) 342–347, https://doi.org/10.1177/104063879400600310.

[5] H. Schäeffler, A. Breitrück, *Clostridium difficile* – from colonization to infection, Front. Microbiol. 9 (2018) 646, https://doi.org/10.3389/fmicb.2018.00646.

[6] K.E. Burke, J.T. Lamont, *Clostridium difficile* infection: a worldwide disease, Gut and liver 8 (1) (2014) 1–6, https://doi.org/10.5009/gnl.2014.8.1.1.

[7] S.L. Revolinski, L.S. Munoz-Price, *Clostridium difficile* in immunocompromised hosts: a review of epidemiology, risk factors, treatment, and prevention, Clin. Infect. Dis. 68 (12) (2019) 2144–2153, https://doi.org/10.1093/cid/ciy845.

[8] S.K. Lim, R.L. Stuart, K.E. Mackin, G.P. Carter, D. Kotsanas, M.J. Francis, M. Easton, K. Dimovski, B. Elliott, T.V. Riley, G. Hogg, E. Paul, T.M. Korman, T. Seemann, T. P. Stinear, D. Lyras, G.A. Jenkin, Emergence of a ribotype 244 strain of *Clostridium difficile* associated with severe disease and related to the epidemic ribotype 027 strain, Clin. Infect. Dis. 58 (12) (2014) 1723–1730, https://doi.org/10.1093/cid/ciu203.

[9] B.-M. Shin, E.Y. Kuak, S.J. Yoo, W.C. Shin, H.M. Yoo, Emerging toxin A-B+ variant strain of *Clostridium difficile* responsible for pseudomembranous colitis at a tertiary care hospital in Korea, Diagn. Microbiol. Infect. Dis. 60 (2008) 333–337, https://doi.org/10.1016/j.diagmicrobio.2007.10.022.

[10] J.S. Weese, Bacterial enteritis in dogs and cats: diagnosis, therapy, and zoonotic potential, Vet. Clin. Small Anim. Pract. 41 (2) (2011) 287–309, https://doi.org/10.1016/j.cvsm.2010.12.005.

[11] S.C. Lim, T.V. Riley, D.R. Knight, One Health: the global challenge of *Clostridium difficile* infection, Microbiology Australia 41 (1) (2020) 23–27, https://doi.org/10.1071/MA20007.

[12] Y. Longtin, B. Paquet-Bolduc, R. Gilca, C. Garenc, E. Fortin, J. Longtin, S. Trottier, P. Gervais, J.F. Roussy, S. Lévesque, D. Ben-David, I. Cloutier, V.G. Loo, Effect of detecting and isolating *Clostridium difficile* carriers at hospital admission on the incidence of *C. difficile* infections. A quasi-experimental controlled study, JAMA Intern. Med. 176 (6) (2016) 796–804, https://doi.org/10.1001/jamainternmed.2016.0177.

[13] N.E. Stone, L.C. Sidak-Loftis, J.W. Sahl, A.J. Vazquez, K.B. Wiggins, J.D. Gillece, N. D. Hicks, J.M. Schupp, J.D. Busch, P. Keim, D.M. Wagner, More than 50% of *Clostridium difficile* isolates from pet dogs in Flagstaff, USA, carry toxigenic genotypes, PLoS One 11 (10) (2016), e0164504, https://doi.org/10.1371/journal.pone.0164504.

[14] D. Rabold, W. Espelage, M. Abu Sin, T. Eckmanns, A. Schneeberg, H. Neubauer, N. Möbius, K. Hille, L.H. Wieler, C. Seyboldt, A. Lübke-Becker, The zoonotic potential of *Clostridium difficile* from small companion animals and their owners, PLoS One 13 (2) (2018), e0193411, https://doi.org/10.1371/jornal.pone.0193411.

[15] K. Rainha, R. Fernandes Ferreira, C.N.R. Trindade, L.G. Carneiro, B. Penna, B. T. Endres, K. Begum, M.J. Alam, K.W. Garey, C.P. Domingues Regina Maria, E. O. Ferreira, Characterization of *Clostridioides difficile* ribotypes in domestic dogs in Rio de Janeiro, Brazil, Anaerobe 58 (2019) 22–29, https://doi.org/10.1016/j.anaerobe.2019.06.007.

[16] J.S. Weese, H.R. Staempfli, J.F. Prescott, S.A. Kruth, S.J. Greenwood, H.E. Weese, The roles of *Clostridium difficile* and enterotoxigenic *Clostridium perfringens* in diarrhea in dogs, J. Vet. Intern. Med. 15 (4) (2001) 374–378, https://doi.org/10.1111/j.1939.1676.2001.tb02332.x.

[17] S.L. Marks, E.J. Kather, P.H. Kass, A.C. Melli, Genotypic and phenotypic characterization of *Clostridium perfringens* and *Clostridium difficile* in diarrheic and healthy dogs, J. Vet. Intern. Med. 16 (5) (2002) 533–540, https://doi.org/10.1892/0891-6640(2002).016<0533:gapcop>2.3.co;2.

[18] C. Orden, J.L. Blanco, S. Álvarez-Pérez, M. Garcia-Sancho, F. Rodriguez-Franco, A. Sainz, A. Villaescusa, C. Harmanus, E. Kuijper, M.E. Garcia, Isolation of *Clostridium difficile* from dogs with digestive disorders, including stable metronidazole-resistant strains, Anaerobe 43 (2017) 78–81, https://doi.org/10.1016/j.anaerobe.2016.12.008.

[19] A.N. Diniz, F.M. Coura, M. Rupnik, V. Adams, T.L. Stent, J.I. Rood, C.A. de Oliveira Jr., F.C.F. Lobato, R.O.S. Silva, The incidence of *Clostridioides difficile* and *Clostridium perfringens* netF-positive strains in diarrheic dogs, Anaerobe 49 (2018) 58–62, https://doi.org/10.1016/j.anaerobe.2017,12.003.

[20] J. Perrin, C. Buogo, A. Gallusser, A.P. Burnens, J. Nicolet, Intestinal carriage of *Clostridium difficile* in neonate dogs, Journal of Veterinary Medicine, Series B 40 (3) (1993) 222–226, https://doi.org/10.1111/j.1439-0450.1993.tb00131.x.

[21] A.S.F. Berry, B.J. Kelly, D. Barnhart, D.J. Kelly, D.P. Beiting, R.N. Baldassano, L. E. Redding, Gut microbiota features associated with *Clostridioides difficile* colonization in puppies, PLoS One 14 (8) (2019) 1–15, 10.137/journal.pone.0215497.

[22] J.S. Weese, *Clostridium (Clostridioides) difficile* in animals, J. Vet. Diagn. Invest. 32 (2) (2020) 213–221, https://doi.org/10.1177/1040638719899081.

[23] T.V. Riley, J.E. Adams, G.L. O'Neill, R.A. Bowman, Gastrointestinal carriage of *Clostridium difficile* in cats and dogs attending veterinary clinics, Epidemiol. Infect. 107 (3) (1991) 659–665, https://doi.org/10.1017/s0950268800049359.

[24] N. Chouicha, S.L. Marks, Evaluation of five enzyme immunoassays compared with the cytotoxicity assay for diagnosis of *Clostridium difficile*-associated diarrhea in dogs, J. Vet. Diagn. Invest. 18 (2) (2006) 182–188, https://doi.org/10.1177/104063870601800207.

[25] S.L. Lefebvre, J.S. Weese, Contamination of pet therapy dogs with MRSA and *Clostridium difficile*, J. Hosp. Infect. 72 (3) (2009) 268–283, https://doi.org/10.1016/j.jhin.2009.02.019.

[26] M. Usui, K. Suzuki, K. Oka, K. Miyamoto, M. Takahashi, T. Inamatsu, S. Kamiya, Y. Tamura, Distribution and characterization of *Clostridium difficile* isolated from dogs in Japan, Anaerobe 37 (2016) 58–61, https://doi.org/10.1016/j.anaerobe.2015.10.002.

[27] J.K. Clooten, S.A. Kruth, J.S. Weese, Genotypic and phenotypic characterization of *Clostridium perfringens* and *Clostridium difficile* in diarrheic and healthy dogs, J. Vet. Intern. Med. 17 (2) (2003) 123, https://doi.org/10.1111/j.1939-1676.2003.tb02421.x.

[28] S.L. Lefebvre, L.G. Arroyo, J.S. Weese, Epidemic *Clostridium difficile* strain in hospital visitation dog, Emerg. Infect. Dis. 12 (6) (2006) 1036–1037, https://doi.org/10.3201/eid1206.060115.

[29] J.S. Weese, P.P. Mshelbwala, F. Lohr, *Clostridium difficile* shedding by healthy dogs in Nigeria and Malawi, Zoonoses and Public Health 66 (6) (2019) 618–621, https://doi.org/10.1111/zph12613.

[30] N. al Saif, J.S. Brazier, The distribution of *Clostridium difficile* in the environment of South Wales, J. Med. Microbiol. 45 (2) (1996) 133–137, https://doi.org/10.1099/00222615-45-2-133.
[31] A. Schneeberg, M. Rupnik, H. Neubauer, C. Seyboldt, Prevalence and distribution of *Clostridium difficile* PCR ribotypes in cats and dogs from animal shelters in Thuringia, Germany, Anaerobe 18 (5) (2012) 484–488, https://doi.org/10.1016/j.anaerobe.2012.08.002.
[32] K.J. Wetterwik, G. Trowald-Wigh, L.L. Fernström, K. Krovacek, *Clostridium difficile* in faeces from healthy dogs and dogs with diarrhea, Acta Vet. Scand. 55 (2013) 23, https://doi.org/10.1186/1751-0147-55-23.
[33] I. Hussain, R.K. Sharma, P. Borah, S. Rajkhowa, I. Hussain, L.M. Barkalita, D. Hasin, M. Choudhury, M. Rupnik, N.K. Deka, G.K. Saikia, Isolation and characterization of *Clostridium difficile* from pet dogs in Assam, India, Anaerobe 36 (2015) 9–13, https://doi.org/10.1016/janaerobe.2015.09.006.
[34] J.S. Weese, J. Armstrong, Outbreak of *Clostridium difficile*-associated disease in a small animal veterinary teaching hospital, J. Vet. Intern. Med. 17 (6) (2003) 813–816, https://doi.org/10.1111/j.1939-1676.2003.tb02519.x.
[35] R.O.S. Silva, R.L.R. Santos, P.S. Pires, L.C. Pereira, S.T. Pereira, M.C. Duarte, R. A. Assis, F.C.F. Lobato, Detection of toxins A/B and isolation of *Clostridium difficile* and *Clostridium perfringens* from dogs in Minas Gerais, Braz. J. Microbiol. 44 (1) (2013) 133–137, https://doi.org/10.1590/S1517-83822013005000008.
[36] R.O.S. Silva, L. Ribeiro de Almeida, C.A. Oliveira Junior, D.F. de Magalhães Soares, P.L.L. Pereira, M. Rupnik, F.C.F. Lobato, Carriage of *Clostridium difficile* in free-living South American coati (Nasua nasua) in Brazil, Anaerobe 30 (2014) 99–101, https://doi.org/10.1016/j.anaerobe.2014.09.012.
[37] WSAVA (World Small Animal Veterinary Association), International Gastrointestinal Standardization Group. Endoscopic, biopsy, and histopathological guidelines for the evaluation of gastrointestinal inflammation in companion animals, J. Vet. Intern. Med. 24 (1) (2010) 10–26, https://doi.org/10.1111/j.1939-1676.2009.0443.x.
[38] J.S. Munday, C.V. Löhr, M. Kiupel. Tumors of the alimentary tract. In: Meuten, Donald J. (Ed.). Tumors in Domestic Animals, 5. ed., Cap. 13, pp.499−601. USA: Wiley-Blackwell. doi:10.1002/9781119181200.ch.13.
[39] K.A. Allenspach, J.P. Mochel, Y. Du, S.L. Priestnall, F. Moore, M. Slayter, A. Rodrigues, M. Ackermann, M. Krochenberger, J. Mansell, , WSAVA GI Standardization Working Group, N. Luckschander, C. Wang, J. Suchodolski, N. Berghoff, A.E. Jergens, Correlating gastrointestinal histopathologic changes to clinical disease activity in dogs with idiopathic inflammatory bowel disease, Veterinary Pathology 56 (3) (2019) 435–443, https://doi.org/10.1177/0300985818813090.
[40] M. Lister, E. Stevenson, D. Heeg, N.P. Minton, S.A. Kuehne, Comparison of culture based methods for the isolation of *Clostridium difficile* from stool samples in a research setting, Anaerobe 28 (2014) 226–229, https://doi.org/10.1016/j.anaerobe.2014.07.003.
[41] I. Poilane, F. Bert, P. Cruaud, M.-H. Nicolas-Chanoine, A. Collingnon, Interest of the disk diffusion method for screening Clostridium difficile isolates with decreased susceptibility to antibiotics, Pathol. Biol. 55 (8−9) (2007) 429–433, https://doi.org/10.1016/j.patbio.2007.07.010.
[42] A.N. Diniz, L.N.F. Moura, D.S.G. Cruz, C.A. Oliveira Junior, H.C.P. Figueiredo, J.L. R. Cunha, E.G. Vilela, E.J. Kuijper, M.H. Wilcox, F.C.F. Lobato, R.O.S. Silva, Characterization of the virulence of three novel clade 2 Clostridioides (Clostridium) difficile strains and a two-year screening in animals and humans in Brazil, PLoS One 17 (8) (2022), e0273013, https://doi.org/10.1371/journal.pone.0273013.
[43] L. Chunhui, C. Harmanus, Z. Duolong, M. Xiujuan, W. Shachui, D. Juping, L. Sidi, F. Chenchao, Z. Pengching, L. Ruisi, W. Anhua, E.J. Kuijper, W.K. Smits, F. Lei, S. Xingmin, Characterization of the virulence of a non-RT027, non-RT078 and binary toxin-positive *Clostridium difficile* strain associated with severe diarrhea, Emerg. Microb. Infect. 7 (1) (2018) 211, https://doi.org/10.1038/s41426-018-0211-1.
[44] V. Pantaléon, A.P. Soavelomandroso, S. Bouttier, R. Briandet, B. Roxas, M. Chu, A. Collignon, C. Janoir, G. Vedantam, T. Candela, The *Clostridium difficile* protease Cwp84 modulates both biofilm formation and cell-surface properties, PLoS One 10 (4) (2015), e0124971, https://doi.org/10.1371/journal.pone.0124971.
[45] D. Wroblewski, G.E. Hannett, D.J. Bopp, G.K. Dumyati, T.A. Halse, N.B. Dumas, K. A. Musser, Rapid molecular characterization of *Clostridium difficile* and assessment of populations of *C. difficile* in stool specimens, J. Clin. Microbiol. 47 (7) (2009) 2142–2148, https://doi.org/10.1128/JCM.02498-08.
[46] P. Bidet, F. Barbut, V. Lalande, B. Burghoffer, J.C. Petit, Development of a new PCR-ribotyping method for *Clostridium difficile* based on ribosomal RNA gene sequencing, FEMS (Fed. Eur. Microbiol. Soc.) Microbiol. Lett. 175 (2) (1999) 261–266, https://doi.org/10.1111/j.1574-6968.1999.tb13629.x.
[47] M.J. Aldape, D.D. Heeney, A.E. Bryant, D.L. Stevens, Tigecycline suppresses toxin A and B production and sporulation in *Clostridium difficile*, J. Antimicrob. Chemother. 70 (1) (2015) 153–159, https://doi.org/10.1093/jac/dku325.
[48] S.P. Borriello, P. Honour, T. Turner, F. Barclay, Household pets as a potential reservoir for *Clostridium difficile infection*, J. Clin. Pathol. 36 (1) (1983) 84–87, https://doi.org/10.1136/jcp.36.1.84.
[49] M.G. Koene, D. Mevius, J.A. Wagenaar, C. Harmanus, M.P. Hensgens, A. M. Meetsma, F.F. Putirulan, M.A. Van Bergen, E.J. Kuijper, *Clostridium difficile* in Dutch animals: their presence, characteristics and similarities with human isolates, Clin. Microbiol. Infect. 18 (8) (2012) 778–784, https://doi.org/10.1111/j.1469-0691.2011.03651.x.
[50] S. Álvarez-Pérez, J.L. Blanco, T. Peláez, M.P.P. Lanzarot, C. Harmanus, E. Kujiper, M.E. García, Faecal shedding of antimicrobial-resistant *Clostridium difficile* strains by dogs, J. Small Anim. Pract. 56 (3) (2014) 190–195, https://doi.org/10.1111/jsap.12311.
[51] J. Clooten, S. Kruth, L. Arroyo, J.S. Weese, Prevalence and risk factors for *Clostridium difficile* colonization in dogs and cats hospitalized in an intensive care unit, Vet. Microbiol. 129 (1–2) (2008) 209–214, https://doi.org/10.1016/j.vetmic.2007.11.013.
[52] S.L. Lefebvre, D. Waltner-Toews, A.S. Peregrine, R. Reid-Smith, L. Hodge, L. G. Arroyo, J.S. Weese, Prevalence of zoonotic agents in dogs visiting hospitalized people in Ontario: implications for infection control, J. Hosp. Infect. 62 (4) (2006) 458–466, https://doi.org/10.1016/j.jhin.2005.09.025.
[53] Q. Tang, G. Jin, G. Wang, T. Liu, X. Liu, B. Wang, H. Cao, Current sampling methods for gut microbiota: a call for more precise devices, Front. Cell. Infect. Microbiol. 10 (2020) 151, https://doi.org/10.3389/fcimb.2020.00151.
[54] M.A. Orellana-Miguel, A. Alcolea-Medina, L. Barrado-Branco, J. Rodriguez-Otero, F. Chaves-Sánchez, Algorithm proposal based on the C. Diff Quik Chek Complete ICT device for detecting *Clostridium difficile* infection, Enferm. Infecc. Microbiol. Clín. 31 (2) (2013) 97–99, https://doi.org/10.1016/j.eimc.2012.01.003.
[55] J.-W. Cheng, M. Xiao, T. Kudinha, Z.-P. Xu, L.-Y. Sun, X. Hou, L. Zhang, X. Fan, F. Kong, Y.-C. Xu, The role of glutamate dehydrogenase (GDH) testing assay in the diagnosis of *Clostridium difficile* infections: a high sensitive screening test and an essential step in the proposed laboratory diagnosis workflow for developing countries like China, PLoS One 10 (12) (2015), https://doi.org/10.1371/journal.pone.0144604.
[56] J. Sandlund, R. Mills, C. Griego-Fullbright, A. Wagner, J. Estis, A.J. Bartolome, A. Almazan, S. Tam, S. Biscocho, S. Abusali, N. Nolan, J.J. Bishop, J. Todd, S. Young, Laboratory comparison between cell cytotoxicity neutralization assay and ultrasensitive single molecule counting technology for detection of *Clostridioides difficile* toxins A and B, PCR, enzyme immunoassays, and multistep algorithms, Diagn. Microbiol. Infect. Dis. 95 (1) (2019) 20–24, https://doi.org/10.1016/j.diagmicrobio.2019.04.002.
[57] K.V. Ota, K.L. McGowan, *Clostridium difficile* testing algorithms using glutamate dehydrogenase antigen and *C. difficile* toxin enzyme immunoassays with *C. difficile* nucleic acid amplification testing increase diagnostic yield in a tertiary pediatric population, J. Clin. Microbiol. 50 (4) (2012) 1185–1188, https://doi.org/10.1128/JCM.05620.11.
[58] N.J. Cave, S.L. Marks, P.H. Kass, A.C. Melli, M.A. Brophy, Evaluation of a routine diagnostic fecal panel for dogs with diarrhea, JAVMA 221 (1) (2002) 52–59, https://doi.org/10.2460/javma.2002.221.52.
[59] S. Álvarez-Pérez, J.L. Blanco, C. Harmanus, E.J. Kuijper, M.E. García, Prevalence and characteristics of *Clostridium perfringens* and *Clostridium difficile* in dogs and cats attended in diverse veterinary clinics from the Madrid region, Anaerobe 48 (2017) 47–55, https://doi.org/10.1016/j.anaerobe.2017.06.023.
[60] R. Pilla, J.S. Suchodolski, The role of the canine gut microbiome and metabolome in health and gastrointestinal disease, Front. Vet. Sci. 6 (2020), https://doi.org/10.3389/fvets.2019.00498 art. 498.
[61] P.W. O'Toole, I.B. Jeffery, Gut microbiota and aging, Science 350 (6265) (2015) 1214–1215, https://doi.org/10.1126/science.aac8469.
[62] A. Bednarska, D. Bursa, R. Podlasin, M. Paciorek, A. Skrzat-Klapaczyńska, D. Porowski, J. Raczynska, J. Pula, D. Krogulec, M. Makowiecki, A. Horban, Advanced age and increased CRP concentration are independent risk factors associated with *Clostridioides difficile* infection mortality, Sci. Rep. 10 (1) (2020), 14681, https://doi.org/10.1038/s41598-020-71466-0.
[63] P. Spigaglia, I. Drigo, F. Barbanti, P. Mastrantonio, L. Bano, C. Bacchin, C. Puiatti, E. Tonon, G. Berto, F. Agnoletti, Antibiotic resistance patterns and PCR-ribotyping of *Clostridium difficile* strains isolated from swine and dogs in Italy, Anaerobe 31 (2015) 42–46, https://doi.org/10.1016/j.anaerobe.2014.10.003.
[64] S. Shapey, K. Machin, K. Levi, T.C. Boswell, Activity of a dry mist hydrogen peroxide system against environmental *Clostridium difficile* contamination in elderly care wards, J. Hosp. Infect. 70 (2) (2008) 136–141, https://doi.org/10.1016/j.jhin.2008.06.008.
[65] P. Vohra, I.R. Poxton, Efficacy of decontaminants and disinfectants against *Clostridium difficile*, J. Med. Microbiol. 60 (2001) 1218–1224, https://doi.org/10.1099/jmm.0.030288-0.
[66] R.O.S. Silva, M. Rupnik, A.N. Diniz, E.G. Vilela, F.C.F. Lobato, *Clostridium difficile* ribotypes in humans and animals in Brazil, Mem. Inst. Oswaldo Cruz 110 (8) (2015) 1062–1065, https://doi.org/10.1590/0074-02760150294.
[67] T. Troiano, C. Harmanus, I.M.J.G. Sanders, V. Pasquale, S. Dumontet, F. Capuano, V. Romano, E.J. Kuijper, Toxigenic *Clostridium difficile* PCR ribotypes in edible marine bivalve molluscs in Italy, Int. J. Food Microbiol. 208 (2015) 30–34, https://doi.org/10.1016/j.ijfoodmicro.2015.05.002.
[68] G.G. Lopes Cançado, R.O.S. Silva, M. Rupnik, A.P. Nader, J.S. Carvalho, G.M. M. Paixão, B.A.M. Resende, F.C.F. Lobato, E.G. Vilela, Clinical epidemiology of *Clostridium difficile* infection among hospitalized patients with antibiotic-associated iarrhea in a university hospital of Brazil, Anaerobe 54 (2018) 65–71, https://doi.org/10.1016/j.anaerobe.2018.08.005.
[69] B.A.P. Roxas, J.L. Roxas, R. Claus-Walker, A. Harishankar, A. Mansoor, F. Anwar, S. Jillella, A. Williams, J. Lindsey, S.P. Elliott, R.W. Shehab, V.K. Viswanathan, G. Vedantam, Phylogenic analysis of *Clostridiodes difficile* ribotype 106 strains reveals novel genetic islands and emergent phenotypes, Sci. Rep. (2020), https://doi.org/10.1038/s41598-020-79123-2.
[70] M.J. Alam, S.T. Walk, B.T. Endres, E. Basseres, M. Khaleduzzaman, J. Amadio, W. L. Musick, J.L. Christensen, J. Kuo, R.L. Atmar, K.W. Garey, Community environmental contamination of toxigenic *Clostridium difficile*. IDSA infections diseases society of America, Open Forum Infect. Dis. 4 (1) (2017) ofx018, https://doi.org/10.1093/ofid.ofx018.

[71] L. Suárez-Bode, R. Barrón, J.L. Pérez, A. Mena, Increasing prevalence of the epidemic ribotype 106 in healthcare facility-associated and community-associated *Clostridioides difficile* infection, Anaerobe 55 (2019) 124–129, https://doi.org/10.1016/j.anaerobe.2018.12.002.

[72] D.A. Secco, L.T. Balassiano, R.F. Boente, K.R. Miranda, J. Brazier, V. Hall, J. dos Santos-Filho, L.A. Lobo, S.A. Nouér, R.M. Domingues, *Clostridium difficile* infection among immunocompromised patients in Rio de Janeiro, Brazil and detection of moxifloxacin resistance in a ribotype 014 strain, Anaerobe 28 (2014) 85–89, https://doi.org/10.1016/j.anaerobe.2014.05.013.

[73] A. Nicholas, Y.K. Kim, W.-K. Lee, G.N. Selasi, S.H. Na, H.I. Kwon, Y.J. Kim, H. S. Lee, K.E. Song, J.H. Shin, J.C. Lee, Molecular epidemiology and antimicrobial susceptibility of *Clostridium difficile* isolates from two Korean hospitals, PLoS One 12 (3) (2017), e0174716, https://doi.org/10.1371/jornal.pone.0174716.

[74] A.N. Diniz, C.A. Oliveira Júnior, E.G. Vilela, H.C.P. Figueiredo, M. Rupnik, M. H. Wilcox, W.N. Fawley, D.S. Blanc, F.C.F. Lobato, R.O.S. Silva, Molecular epidemiology of *Clostridioides* (previously *Clostridium) difficile* isolates from a university hospital in Minas Gerais, Brazil, Anaerobe 56 (2019) 34–39, https://doi.org/10.1016/j.anaerobe.2019.01.010.

[75] S. Janezic, V. Zidaric, B. Pardon, A. Indra, B. Kokotovic, J.L. Blanco, C. Seyboldt, C. R. Diaz, I.R. Poxton, V. Perreten, I. Drigo, A. Jiraskova, M. Ocepek, J.S. Weese, J. G. Songer, M.H. Wilcox, M. Rupnik, International *Clostridium difficile* animal strain collection and large diversity of animal associated strains, BMC Microbiol. 14 (2014) 73, https://doi.org/10.1186/1471-2180-14-173, art.1.

[76] A.N. Diniz, R.O.S. Silva, C.A. Oliveira Jr., F. Pierezan, F.C.F. Lobato, *Clostridium perfringens* type A netF and netE positive and *C. difficile* co-infection in two adult dogs, Anaerobe 38 (2016) 94–96, https://doi.org/10.1016/j.anaerobe.2015.12.013.

[77] I.T. Balassiano, K.R. Miranda, R.F. Boente, H. Pauer, I.C. Oliveira, J. Santos-Filho, E.L. Amorim, G.A. Caniné, C.F. Souza, M.Z. Gomes, E.O. Ferreira, J.S. Brazier, R. M. Domingues, Characterization of *Clostridium difficile* strains isolated from immunosuppressed inpatients in a hospital in Rio de Janeiro, Brazil, Anaerobe 15 (3) (2009) 61–64, https://doi.org/10.1016/j.anaerobe.2008.12.007.

[78] C. Albuquerque, D. Pagnossin, K. Landsgaard, J. Simpson, D. Brown, J. Irvine, D. Candlish, A.E. Ridyard, G. Douce, C. Millins, The duration of antibiotic treatment is associated with carriage of toxigenic and 2 atoxigenic strains of *Clostridioides difficile* in dogs, PLoS One 16 (5) (2021), e0245949, https://doi.org/10.1371/jornal.pone.0245949.

[79] C.N.R. Trindade, R.M.C.P. Domingues, E.O. Ferreira, The epidemiology of *Clostridioides difficile* infection in Brazil: a systematic review covering thirty years, Anaerobe 58 (2019) 13–21, https://doi.org/10.1016/j.anaerobe.2019.03.002.

[80] M.S.M. Brouwer, A.P. Roberts, H. Hussain, R.J. Williams, E. Allan, P. Mullany, Horizontal gene transfer converts non-toxigenic *Clostridium difficile* strains into toxin producers, Nat. Commun. 4 (2013) 2601, https://doi.org/10.1038/ncomms3601.

[81] R.E. Goyette, N.S. Key, E.W. Ely, Hematologic changes in sepsis and their therapeutic implications, Semin. Respir. Crit. Care Med. 25 (6) (2004) 645–659, https://doi.org/10.1055/s-2004-860979.

[82] A.S. Chaudhry, B.N. Azab, J.T. McGinn, S.W. Bloom, Neutrophil lymphocyte ratio vs total leukocyte count as a predictor of adverse outcomes in *Clostridium difficile* associated diarrhea (CDAD), J. Am. Coll. Surg. 221 (4) (2015) Suppl–2, https://doi.org/10.1016/j.jamcollsurg.2015.08.063.

[83] S.L. Marks, S.C. Rankin, B.A. Byrne, J.S. Weese, Enteropathogenic bacteria in dogs and cats: diagnosis, epidemiology, treatment, and control, J. Vet. Intern. Med. 25 (6) (2011) 1195–1208, https://doi.org/10.1111/j.1939.1676.2011.00821.x.

[84] D. Griffiths, W. Fawley, M. Kachrimanidou, R. Bowden, D.W. Crook, R. Fung, T. Golubchik, R.M. Harding, J.F. Jeffery, K.A. Jolley, R. Kirton, T.E. Peto, G. Rees, N. Stoesser, A. Vaughan, A.S. Walker, B.C. Young, M. Wilcox, K.E. Dingle, Multilocus sequence typing of *Clostridium difficile*, J. Clin. Microbiol. 48 (3) (2010) 770–778, https://doi.org/10.1128/JCM.01796-09.