



Clostridium difficile beyond stools: dog nasal discharge as a possible new vector of bacterial transmission



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ABSTRACT

Zoonotic transmission of *Clostridium difficile* has been largely hypothesised to occur after direct or indirect contact with contaminated animal faeces. Recent studies have reported the presence of the bacterium in the natural environment, including in soils and rivers. If *C. difficile* spores are scattered in the environment, they can easily enter the respiratory tract of dogs, and therefore, dog nasal discharge could be a direct route of transmission not previously investigated. This study reports for the first time the presence of *C. difficile* in the respiratory tracts of dogs. The bacterium was isolated from 6 (17.1%) out of 35 nasal samples, with a total of 4 positive dogs (19%). *C. difficile* was recovered from both proximal and distal nasal cavities. All isolates were toxigenic and belonged to PCR-ribotype 014, which is one of the most predominant types in animals and in community-acquired *C. difficile* infections in recent years. The findings of this study demonstrate that the nasal cavity of dogs is contaminated with toxigenic *C. difficile*, and therefore, its secretions could be considered as a new route by which bacteria are spread and transmitted.

1. Introduction

Clostridium difficile (*C. difficile*), recently renamed as *Clostridioides difficile* (Lawson et al., 2016), is the most important cause of nosocomial infectious diarrhoea worldwide. Considered an enteric pathogen, the main risk factor for bacterial colonisation is a disruption of the normal gut microbiota, frequently due to the exposure to broad-spectrum antibiotics (Lübbert et al., 2018). The spread and severity of *C. difficile* infection (CDI) have been associated with specific strains, including PCR-ribotypes 078, 027 014 and 020, among others. The prevalence rates of which have increased in both Europe and North America between 2012 and 2015 (Cheknis et al., 2018). Transmission of the bacterium occurs via the faecal-oral route, directly or indirectly (Lübbert et al., 2014), and the spores are considered the main form implicated in *C. difficile* transmission and infection. They have the ability to persist for months on environmental surfaces, while the vegetative cells of

C. difficile die in hours in aerobic conditions and they are normally killed with acid gastric exposure (Jump et al., 2007). The infectious dose in humans is not clearly described and it appears to be low and to vary with the antibiotic class or dose previously used (Warriner et al., 2017; Best et al., 2012).

C. difficile is also an important enteric pathogen in farm animals and pets, causing acute diarrhoea and colitis (Baverud, 2002). This animal reservoir has been linked with the increased number of community-associated infections, as the same strains have been observed in both humans and animals simultaneously (Rodriguez et al., 2016). Recently, dogs and cats have attracted the attention of many researchers since pets are in continuous close contact with their owners and often share the same resting places at home (bedrooms, living rooms, kitchens). Previous studies have demonstrated that shoes, slippers and dog paws could spread *C. difficile* spores among the environment, humans and animals (Janezic et al., 2018; Lefebvre and Weese, 2009). Most

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recently, a systematic large-scale survey was conducted for the first time to determine the occurrence of *C. difficile* in dogs and cats and their owners, and a bacterium prevalence of 3% was observed in faecal samples from both pets and humans, with PCR-ribotype 014 as the predominant type (Rabold et al., 2018).

Since the discovery of *C. difficile* in 1935 (Hall and O'Toole, 1935), studies have investigated the bacterium in biological faecal samples or in the environment (different surfaces, water or air samples) (Rodriguez Diaz et al., 2018), and it has been demonstrated that soils are loaded with *C. difficile* spores (Dharmasena and Jiang, 2018; Rodriguez Diaz et al., 2018). Dogs are likely to smell everything that is in their reach to analyse their surroundings. Therefore, if *C. difficile* spores are scattered in the environment, the noses of dogs should be the first place where the spores enter, and the nasal discharge, seemingly harmless, could be a more direct route of spore transmission than faeces. *C. difficile* transmission could occur after exposure to kisses and other licks, secretions and sneezes, specially in those dogs that are in close and continuous contact with their owners. The objective of this study is to demonstrate for the first time the presence of *C. difficile* in the respiratory tract of dogs and to perform a molecular characterisation of the isolates. This study will finally reveal a new route of *C. difficile* transmission.

2. Methods

2.1. Dog recruitment and sample collection

A prospective study was conducted over three months at the Small Companion Animals Clinic, Department of Companion Animals, Faculty of Veterinary Medicine of Liege, from April through June 2017. Respiratory tract swab samples and bronchoalveolar lavage fluid were collected from each dog enrolled in the study. Eligible dogs were animals in consultation or those staying in the hospital who had respiratory troubles. Eight additional samples were obtained from the Faculty of Veterinary Medicine, University of Helsinki. Blank controls of all types of samples were obtained to verify possible contaminations.

Data related to clinical history, diagnostic findings and treatment received were collected. In addition, all predisposing factors for developing *C. difficile* infection (CDI), such as the prescription of antimicrobials, were carefully recorded. The study was examined and approved by the Animal Faculty Ethics Committee of the University of Liège (16–1854).

Animals were anaesthetised for sample collection. Before the anaesthesia, dogs were fasted the same morning, with access only to fresh water. Clinical examination was performed to ensure the healthy status of the dog. A catheter was placed in the cephalic vein for simultaneous collection of blood via the blood test catheter, to ensure the healthy status of the animal. Then, the premedication was injected (butorphanol 0.3 mg/kg IV) with a subsequent wait time of 20 minutes (duration for the implementation of the premedication). After this period, the anaesthesia was induced with midazolam 0.2 mg/kg IV and IV propofol on demand. The dog was not intubated and breathed ambient air. The endoscope was inserted directly through the larynx into the trachea, taking care to minimise contamination of the final part when passing through the oral cavity. A sterile swab was introduced to brush the mucosa at the proximal nasal cavity. A second sterile swab was introduced to brush the mucosa at the distal third of the nasal cavity, after opening the nare using a sterile speculum. The top of the swabs were cut, stored in sterile cryotubes and banked at -4 °C. Bronchoalveolar lavage fluid was obtained following the method described by Ericsson et al. (2016) with minor modifications. When samples were not processed the same day, they were stored at -80 °C, otherwise they were stored at -4°C. The dog was awoken under surveillance with monitoring of its body temperature (as hypothermia was possible following the anaesthesia). The catheter was removed once the dog was fully awake. The anaesthesia was supervised by a veterinarian with extensive expertise in anaesthesiology. Pulse oxymetry and ECG (electrocardiography) were used to monitor all interventions. Equipment

for intubation and ventilation of dogs was available in cases of secondary apnea caused by the induction of anaesthesia. Swab controls from the endoscope were taken to determine possible contaminations during the intervention.

2.2. *C. difficile* culture and identification

Samples were cultured according to a protocol for *C. difficile* detection based on the work of Delmée et al. (2005). A total of 300 µl of buffered peptone water was added to the cryotube containing the swab sampled or the bronchoalveolar lavage fluid. The sample was mixed by vortex for 1 minute and then incubated for 5 minutes at room temperature. Subsequently, 100 µl of the broth was spread on cycloserine cefoxitin fructose agar with taurocholate (CCFAT) (Delmée et al., 1987). Plates were incubated in an anaerobic workstation, Concept Plus (Led Techno, BE), for *C. difficile* direct detection. The remaining sample volume, around 200 µl minimum, was recovered and inoculated into 10 ml of cycloserine cefoxitin fructose taurocholate broth (CCFT) (Delmée et al., 1987), which was freshly prepared in the laboratory. The broth was incubated for 72 h at 37 °C under anaerobic conditions. After incubation, a 10 µl aliquot of the enriched broth was spread onto CCFAT, and these plates were incubated anaerobically at 37 °C for 2 days, for *C. difficile* detection after enrichment. Colonies of *C. difficile* were identified from culture plates by morphological criteria, subcultured onto blood agar (5% sheep blood; Bio-Rad, BE) and checked using a rapid *C. difficile* latex agglutination test kit (DR 1107A, Oxoid, FR). The toxin activity of the isolates was confirmed by a cytotoxicity assay using confluent monolayer MRC-5 cells as described previously (Rodriguez et al., 2013). Confirmation of *C. difficile* was performed by detection of a species-specific internal fragment of the *tpi* gene and detection of genes for toxin A, toxin B and binary toxin (*cdtA*) by classic PCR (Rodriguez et al., 2013).

2.3. PCR-ribotyping

PCR-ribotyping was performed as described previously (Bidet et al., 1999). The technique is based on capillary gel electrophoresis. International nomenclature was used for *C. difficile* strains that presented a PCR-ribotype profile matching the Cardiff PCR-ribotypes (Anaerobe Reference Unit (ARU), UK) from the strain collection available in our laboratory. Otherwise, strains were identified with an internal nomenclature beginning with UCL (database at Catholic University of Louvain, National Reference Laboratory for *C. difficile* in humans in Belgium). Strains were identified as rare profiles if they presented new PCR-ribotype profiles rarely or never before detected in our laboratory.

3. Results

Overall, 35 samples were analysed from 21 different dogs. *C. difficile* was isolated from 6 (17.1%) out of 35 samples, with a total of 4 animals positive for the bacterium (19%). The highest proportion of dogs (10 out of 21; 47.6%) was aged between 3 and 8 years old, whereas 7 dogs out of 21 were younger than 3 years old (33.3%). Surprisingly, all of the dogs that were positive for *C. difficile* belonged to the last group with the youngest ages, ranging between 1 and 3 years old (Tables 1 and 2). Dogs that were positive for *C. difficile* were detected among the animals suffering brachiocephalic airway obstructive syndrome (n = 3) and stress intolerance (n = 1).

None of the samples were positive by direct culture. Positive samples were only detected after three days of selective enrichment. One positive animal presented the bacterium in both nostrils (left and right). In another positive animal, *C. difficile* was isolated from both the proximal and distal nasal cavities. The bacterium was isolated from a third positive dog only from the right proximal nasal cavity, but *C. difficile* was not isolated from the left side or from the bronchoalveolar lavage fluid (Table 2).

Only one PCR-ribotype was identified among the 6 positive samples found. The isolates had a profile that corresponded with the ARU Cardiff collection PCR-ribotype 014. All of these isolates displayed cytotoxic activity in cultured cells and contained *tcdA* and *tcdB* genes. However, none of them presented *cdtA* and *cdtB* genes coding for the binary toxin CDT (Table 2).

4. Discussion

Carriage of *C. difficile* in domestic animals and its impact have been a major concern in the last decade. Several studies have underlined the presence of hypervirulent strains in farm animals and pets and their potential association with the increase in community-associated CDI (Rodriguez Diaz et al., 2018). The suggested routes of spore contamination include direct or indirect contact with animal faeces (Rodriguez et al., 2016). In addition to animal carriage, an important reservoir of toxigenic strains has recently been observed in the environment, with the presence of *C. difficile* spores in effluents, rivers, public parks and farmlands. These findings enhance the important role of dog paws and even shoes in *C. difficile* spread, but also suggest that there must be other vectors for the bacterium transmission (Zidaric et al., 2010). Most of the studies reported low levels of *C. difficile* spores in foods, animals or in the environment, as the bacterium was only detected after anaerobic enrichment. However, as the infectious dose and risk factors for CDI in the community are not fully understood, it has been suggested that continuous exposure to these contamination sources over days, weeks or

even years can finally trigger the infection (Rupnik, 2010; Weese et al., 2009).

The noses of most domestic and farm animals are in constant contact with the ground. In the case of dogs, their extraordinary olfactory capability allows them to detect a vast number of chemical compounds, to detect a target odour among a myriad of odours in the environment and to communicate with other congeners (Jenkins et al., 2018). If dogs constantly smell the entire environment, including urine or faeces from other animals, their nasal cavities are exposed to *C. difficile* spores, and the bacterium could find a transient niche in this mucosa. Furthermore, dogs are continuously licking their noses. If the nasal discharge is contaminated, the spores can be disseminated in the environment or they can easily reach the intestinal tract of the dog by oral route. Once they are in the intestinal tract, these spores could trigger the infection or be transient. In any case, they will be excreted with the faeces, contributing to the dissemination in the environment and repeating the cycle of transmission.

This study demonstrates for the first time the presence of toxigenic *C. difficile* strains in the proximal and distal nasal cavities of dogs. *C. difficile* was isolated from the noses of dogs with a percentage of 19% by classical culture and after enrichment. The prevalence reported in adult dog paws and their households does not exceed 34% (Janezic et al., 2018). Therefore, the preliminary findings of this study indicate that animal nasal cavities merit further attention regarding the spread of *C. difficile* and the microbiota composition. It is important to highlight that dogs are very social pack animals. Therefore dog-to-dog

Table 1

Detailed clinical data of dogs enrolled in the study.

Origin	Animal ID	Breed	Genre	Weight (Kg)	Hospitalization/ Consultation	Date of naissance	Consultation/illness	Medical treatment ^a
Belgium	AA	French Bulldog	Female	10	Consultation	19.11.2013	BAOS, Small nostrils Breathing difficulties	NTR
	AB	French Bulldog	Male	11	Consultation	02.12.2014	BAOS, Breathing difficulties	NTR
	AC	Malinois	Male	30 approx.	Consultation	14.07.2014	Stress intolerance	Biclar 500mg 1x/day ^b
	AD	French Bulldog	Male	15	Consultation	24.03.2015	BAOS, Post-operative consultation	Sedacid 20 mg: ¼ tablet BID ^b
	AE	French Bulldog	Female	11	Consultation	13.05.2016	BAOS	NTR
	AF	French Bulldog	Male	11 approx.	Consultation	06.03.2016	Cough	Omeprazole 1 mg/kg SID ^b Magaldrate ½ sachet BID ^b
	AG	Free-ranging dog	Male	38	Consultation	27.04.2004	Laryngeal paralysis	Both of them for 3 weeks Dexamethasone SC Codeine PO ^b
	AH	Westie	Female	10.1	Consultation	03.05.2004	Pulmonary fibrosis	Amoxycyclav for 2 weeks ^c
	AI	French Bulldog	Male	10 approx.	Consultation	01.10.2012	BAOS	Prednisolone 0.5 mg/Kg BID ^c
	AJ	Free-ranging dog	Female	NAD	Consultation	21.06.2011	Sneeze	Amoxycyclav for 3 weeks ^c
	AK	Border Collie	Male	17	Consultation	01.03.2008	Cordvocalectomy control	Prednisolone 0.5mg/Kg BID ^b Xanax 0.1–0.01 mg/kg SID ^b
	AL	French Bulldog	Male	10.7	Consultation	01.01.2013	BAOS	Omeprazole 10 mg/kg BID ^b
	AM	French Bulldog	Male	10.5	Hospitalization 1 day	01.01.2010	BAOS	NAD
Finland	BA	Westie	Male	10 approx.	Consultation	10.01.2009	Idiopathic pulmonary fibrosis	NAD
	BB	Westie	Male	10 approx.	Consultation	18.11.2009	Idiopathic pulmonary fibrosis	NAD
	BC	Westie	Female	10 approx.	Consultation	28.01.2009	Idiopathic pulmonary fibrosis	NAD
	BD	Westie	Male	10 approx.	Consultation	19.05.2011	Idiopathic pulmonary fibrosis	NAD
	BE	Westie	Male	10 approx.	Consultation	18.05.2017	Idiopathic pulmonary fibrosis	NAD
	BF	Westie	Female	10 approx.	Consultation	21.05.2014	Idiopathic pulmonary fibrosis	NAD
	BG	Westie	Male	10 approx.	Consultation	18.11.2009	Idiopathic pulmonary fibrosis	NAD
	BH	Westie	Male	10 approx.	Consultation	16.10.2008	Idiopathic pulmonary fibrosis	NAD

Animal ID: Animal identification; Malinois: Belgian Shepherd Malinois; Westie: West Highland white terrier; BAOS: Brachycephalic airway obstructive syndrome. NTR: not treatment registered; NDA: not available data; BID: *bis in die* (two times per day); SID: *sem' el in di'e* (once a day).

^a Antibiotic treatment, anti-acid treatment or others.

^b Treatment at the moment of sampling.

^c Treatment between 2 months and 2 weeks before sampling.

Table 2Detailed information about the positive and negative samples for *C. difficile* and molecular characterization of the isolates.

Animal ID	Sampling date	Type of sample	<i>C. difficile</i> detection	PCR-Ribotype	Cytotoxic activity	Toxin Profile		
						<i>tcdA</i>	<i>tcdB</i>	<i>CDT</i>
AA	18.04.2017	Left proximal nasal cavity	ND	–	–	–	–	–
AB	18.04.2017	Right proximal nasal cavity	Positive	014	+	+	+	–
		Left proximal nasal cavity	Positive	014	+	+	+	–
AC	25.04.2017	Right proximal nasal cavity	Positive	014	+	+	+	–
		Right distal third of the nasal cavity	Positive	014	+	+	+	–
AD	27.04.2017	Right proximal nasal cavity	Positive	014	+	+	+	–
		Left proximal nasal cavity	ND	–	–	–	–	–
AE	27.04.2017	Left proximal nasal cavity	Positive	014	+	+	+	–
AF	02.05.2017	Right proximal nasal cavity	ND	–	–	–	–	–
		Left proximal nasal cavity	ND	–	–	–	–	–
AG	03.05.2017	Left proximal nasal cavity	ND	–	–	–	–	–
		Left distal third of the nasal cavity	ND	–	–	–	–	–
AH	05.05.2017	Right proximal nasal cavity	ND	–	–	–	–	–
		Left proximal nasal cavity	ND	–	–	–	–	–
AI	16.05.2017	Left proximal nasal cavity	ND	–	–	–	–	–
		Left distal third of the nasal cavity	ND	–	–	–	–	–
AJ	06.06.2017	Left proximal nasal cavity	ND	–	–	–	–	–
		Left distal third of the nasal cavity	ND	–	–	–	–	–
AK	06.06.2017	Left proximal nasal cavity	ND	–	–	–	–	–
		Left distal third of the nasal cavity	ND	–	–	–	–	–
AL	06.06.2017	Left proximal nasal cavity	ND	–	–	–	–	–
		Left distal third of the nasal cavity	ND	–	–	–	–	–
AM	13.06.2017	Right proximal nasal cavity	ND	–	–	–	–	–
		Right distal third of the nasal cavity	ND	–	–	–	–	–
BA	22.06.2017 ^a	Bronchoalveolar lavage fluid (right side)	ND	–	–	–	–	–
BB	22.06.2017 ^a	Bronchoalveolar lavage fluid (left side)	ND	–	–	–	–	–
BC	22.06.2017 ^a	Bronchoalveolar lavage fluid (right side)	ND	–	–	–	–	–
BD	22.06.2017 ^a	Bronchoalveolar lavage fluid (left side)	ND	–	–	–	–	–
BE	22.06.2017 ^a	Bronchoalveolar lavage fluid (right side)	ND	–	–	–	–	–
		Bronchoalveolar lavage fluid (left side)	ND	–	–	–	–	–
BF	22.06.2017 ^a	Bronchoalveolar lavage fluid (right side)	ND	–	–	–	–	–
		Bronchoalveolar lavage fluid (left side)	ND	–	–	–	–	–
BG	22.06.2017	Bronchoalveolar lavage fluid (right side)	ND	–	–	–	–	–
		Bronchoalveolar lavage fluid (left side)	ND	–	–	–	–	–
BH	22.06.2017	Bronchoalveolar lavage fluid (right side)	ND	–	–	–	–	–

ND: No detection.

^a Samples received from other external clinic at –80 °C. Date of sample defrosting and analysis.

transmission (direct via social interactions) is a likely route of spore transmission. Furthermore, dogs can expose their respiratory tracts directly from smelling faeces or even by investigating the terminal end of their own digestive tracts, which is a common behavior in these animals.

A previous study showed that the canine nasal cavity is inhabited by a highly species-rich bacterial community that is different between healthy dogs and those with disease, and the *Clostridiales* order was identified in the canine nasal cavity by 16S RNA sequencing analysis (Tress et al., 2017). A further next-generation sequencing study of the early life faecal and nasal microbiota of pigs detected *Clostridium* as one of the most dominant genera in both niches during the pre-wearing phase (Slifner et al., 2015). However, their role in the pathophysiology of animal disease is still unclear, as well as their role in bacterial dissemination and transmission.

An unexpected finding was that all of the positive isolates belonged to the same PCR-ribotype. We discarded contaminations during sampling or laboratory analysis, as all of the controls effectuated were negative as well as samples from other dogs examined at the same time. We observed that all of the positive samples were detected during ten consecutive days. Even if a larger sample size would probably provide a great variety of PCR-ribotypes, we hypothesised different theories to explain these findings. The first is that dogs could be directly contaminated in the clinics, waiting rooms, or environment. In fact, this veterinary clinic is located in a privileged place surrounded by small garden areas where dogs usually walk to relax before their consultation. If one dog discharged substantial levels of *C. difficile* spores in its faeces during these days, it is likely that other dogs were contaminated if they encountered these faeces. However further whole genome sequencing analysis is

necessary to elucidate if there is clonality between the isolated strains. Previously, it was also reported that *C. difficile* spores survive up to 5 months on inanimate surfaces (Claro et al., 2014) and in different environments (Dharmasena and Jiang, 2018), which makes this first hypothesis plausible. A second possibility, but not exclusive of the first, is that some PCR-ribotypes could be better adapted to some animals and environments (nature or even animal nasal cavities), which would also explain the uniformity of the types found. In a previous study investigating the presence of *C. difficile* in recreational sandboxes for children and dogs, 8 of the 20 identified isolates belonged to PCR-ribotype 014 (Orden et al., 2018). In other environmental studies, PCR-ribotype 014 was also identified as predominant (16.2% of all isolates) (Zidaric et al., 2010). Furthermore, the highest sporulation rates were observed in these strains after 24 hours (Zidaric and Rupnik, 2016). The adaptability of this type of bacterium to different environments and whether there is an advantage to colonising the canine nasal cavity needs further investigation.

5. Conclusions

Generally, people wash and even disinfect their hands quickly after contact with faeces. However, it is uncommon to see this behaviour in the owners after touching the nose or the nasal discharge of their pets. In this study, we have demonstrated that the nasal cavity of dogs can be contaminated with *C. difficile* spores, and therefore its secretions could be considered as a new vector for transmission not previously described. The situation in other animal species as well as the impact not only for transmission but also for the host merits future investigations.

Declarations

Author contribution statement

C Rodriguez: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

B Taminiau: Conceived and designed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data.

MM Rajamäki: Performed the experiments; Contributed reagents, materials, analysis tools or data.

L Bouchafa: Performed the experiments; Contributed reagents, materials, analysis tools or data.

S Romijn, J Van Broeck: Contributed to sampling collection, reagents, materials, analysis tools or data.

M Delmée: Performed the experiments; Contributed reagents, materials, analysis tools or data.

C Clercx: Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data.

G Daube: Conceived and designed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

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Competing interest statement

The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

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