The Occurrence of *Clostridium difficile* in Different Animal Species in Egypt

ABSTRACT

Clostridium difficile is an anaerobic Gram-positive endospore forming bacterium that is the most substantial cause of nosocomial acquired infection in humans. In addition to the nosocomial dissemination, farm and companion animals are a possible source of human *C. difficile* infection either by direct or indirect contact, environmental contamination, or food infection. The present study was conducted to evaluate the prevalence of *C. difficile* and its toxins in different animal species within the veterinary clinics in Egypt. A total of 249 fecal samples including apparently healthy and diseased animals were collected from 60 cows; 26 buffaloes; 24 sheep; 30 goats; 40 horses; 21 dogs and 30 cats.

The samples were evaluated for the occurrence of *C. difficile* using culture method. All positive *C. difficile* isolates were screened for the presence of toxin A and B toxin genes.

C. difficile was detected in both apparently and diseases animals even though in goats *C. difficile* could be detected neither in apparently healthy nor animals showing clinical signs. In addition, no *Clostridium difficile* has been recovered from apparently healthy Buffaloes and cat. Toxigenic *C. difficile* was detected from ten isolates in apparently healthy animals and eleven isolates from diseased animals. All isolates contained tcdB and tcdA.

The occurrence of other toxicgenic *C. difficile* strains indicates the public health significance.

Introduction:

Clostridium difficile is a spore forming Gram positive strictly anaerobic bacterium that causes hospital and antimicrobial-associated intestinal disease in humans and some animal species ranging from asymptomatic colonization to diarrhea and colitis (Warny *et al*., 2005; McFarland, 2008). *C. difficile* produce two exotoxins A and B and/or a binary toxin, leading to *C. difficile* infection (CDI) symptoms (Clark and Wiselka, 2008; Heinlen and Ballard, 2010). *C. difficile* infection (CDI) predominantly affect elderly persons and may be life threatening (Warny *et al.*, 2005). A remarkable change in the epidemiology of CDI has been encountered over the past recent years, with more than double increase in the incidence, severity, and recurrence rates in humans compared to the early 2000s (McFarland, 2008; CDCP 2005; Juneau *et al*., 2013; Indra et al., 2009; Zilberberg *et al*., 2008). Additionally, there are increasing reports of community-associated CDI, including disease in young individuals and people with few or no conventional risk factors. Interestingly, community-associated CDI cases were linked with the emergent hypervirulent antimicrobial-resistant strains, called epidemic PCR ribotypes 027 and 078, which present also in livestock (Warny *et al*., 2005; CDCP, 2008; Jhung et al., 2008; Kuntz *et al*., 2011; Rupnik, 2007; Songer *et al*., 2009).

The source of infection for community-associated cases of CDI remains uncertain (Weese *et al*., 2010a; Jhung *et al.*, 2008). As *C. difficile* is a ubiquitous bacterium inhabiting the environment and can colonize the intestinal tract of both humans and animals, recent prevalence studies suggested that besides the nosocomial dissemination, farm and companion animals are a potential source of human CDI, either by direct or indirect contact, environmental contamination, or food infection (Hoover and Rodriguez-Palacios 2013; Rodriguez-Palacios *et al*. 2013; Wetterwik *et al*., 2013; Álvarez-Pérez *et al*., 2015; Rabold *et al*., 2018; Koene *et al*., 2012; Avbersek *et al.,* 2009).

Since domestic livestock and companion animals frequently tested positive for toxigenic *C. difficile*, even without displaying any clinical signs, it looks probable that *C. difficile* could be zoonotic. Therefore, animals could play an essential role as carriers of the bacterium (Rodriguez *et al*., 2016).

Therefore, the aim of this study was to determine the prevalence of *C. difficile* and its toxins in different animal species within the veterinary clinics and different localities in Egypt.

Material and methods:

1-Samples:

A total of 249 fecal samples were collected from apparently healthy and diseased animals (60 cows; 26 buffaloes; 24 sheep; 30 goats; 40 horses; 21 dogs; 30 cats) (Table 1) raised in Cairo, Giza, Ismailia and El-fayoum governorates in Egypt, during the period between January 2015 to June 2016.

Table 1: The number of fecal samples collected from apparently healthy and diseased domestic and companion animals:

Animal species	Apparently healthy	Diseased animals	Total
Cows	24	36	60
Buffaloes	15	11	26
Sheep	14	28	42
Goat	3	27	30
Horse	40	-	40
Dogs	21	-	21
Cats	13	17	30

All samples were transferred in icebox to the laboratory of the department of Zoonoses, Faculty of Veterinary Medicine, Cairo University, Egypt, with minimum delay for bacteriological examination.

<u>2- Culturing for *C. difficile*:</u>

Fecal samples were cultured for isolation of *C. difficile*, as described in **Avbersek** *et al*.**, 2009**.

<u>2- Molecular characterization of isolates:</u>

Suspected colonies of *C. difficile* were sub-cultured onto blood agar plates and incubated anaerobically at 37°C/24 h. DNA extraction was extracted by transferring 3-5 colonies into 100 µl of sterile distilled water, heating at 95°C for 3 min and then the suspension was centrifuged at 10000 × g for 5 min. The supernatant was used as a DNA-template.

PCR detection of genes encoding triose phosphate isomerase (tpi), toxin A and B (tcd A and tcd B, respectively) as described by Lemee *et al*. [18]; Stubbs *et al*. [19].

<u>3- Statistical analysis:</u>

Infection rates were compared by the χ2 and Fisher's Exact test. Data analysis was performed using PASW Statistics, Version 18.0 software (SPSS Inc., Chicago, IL, USA). A P-value < 0.05 was considered statistically significant.

Results:

Infection rates in animals:

In cows (*n* = 60) *C. difficile* was detected in 4.17% (1/24) of apparently healthy cows, and in 5.56% (2/36) of cows showing clinical signs. While in buffaloes (*n* = 26), *C. difficile* could only be detected in 9.09% (1/11) of animals showing clinical signs (Table 2). In sheep (*n* = 42) *C. difficile* was detected in 14.29% (2/14) of apparently healthy sheep, and in 14.29% (4/28) of sheep showing clinical signs. While in goats (*n* = 30) , *C. difficile* could be detected neither in apparently healthy nor animals showing clinical signs (Table 2). In apparently healthy horses (*n* = 40), *C. difficile* could be detected in 10% (4/40) of examined animals (Table 2).

In apparently healthy dogs (*n* = 21), *C. difficile* was detected in 14.29% (3/21) of examined animals. While in cats (*n* = 30), *C. difficile* could only be detected in 23.53% (4/17) of animals showing clinical signs (Table 2). The results of molecular characterizing of the ten *C. difficile* isolates from apparently healthy animals and eleven isolates from the diseased one are presented in Table 2. Interestingly, all isolates contained tcdB and tcdA.

The infection rates of *C. difficile* in different animal species didn't differ significantly (*P* > 0.05), suggesting that *C. difficile* recovery was not associated with the animal species.

Table 2: Rate of *C. difficile* infection in the examined apparently healthy and clinically diseased domestic and companion animals (N= 249):

	Apparently healthy animals			Diseased animals		
	Examined samples	Positive cases No.	Positive cases %	Examined samples	Positive cases No.	Positive cases %
Cows	24	1	4.17	36	2	5.56
Buffaloes	15	0	0.00	11	1	9.09
Sheep	14	2	14.29	28	4	14.29
Goat	3	0	0.00	27	0	0.00
Horses	40	4	10.00	-	-	-
Dogs	21	3	14.29	-	-	-

Cats	13	0	0.00	17	4	23.53

Table 3: Molecular characterization *C. difficile* strains isolated from different animals.

	Apparently healthy animals		Diseased animals	
	Examined samples	Toxin typing	Examined samples	Toxin typing
Cows	1	tcdA+ tcdB+	2	tcdA+ tcdB+
Buffaloes	0	-	1	tcdA+ tcdB+
Sheep	2	tcdA+ tcdB+	4	tcdA+ tcdB+
Goat	0	-	0	-
Horses	4	tcdA+ tcdB+	-	-
Dogs	3	tcdA+ tcdB+	-	-
Cats	0	-	4	tcdA+ tcdB+
Total	10	tcdA+ tcdB+	11	tcdA+ tcdB+

Discussion:

This study provides insights into the epidemiology of *C. difficile* in both farm and companion animals.

Cows and buffaloes: Low rates of infection with *C. difficile* in apparently healthy and diseased cows were observed (4.17% and 5.56%; respectively), while a 9.09% infection rate in diseased buffaloes was recorded. Dahms *et al*., 2014 stated that the pathogenicity of *C. difficile* for cattle and calves, is still questionable and that diarrhea was found to be associated mostly with toxin producing strains. Rodriguez *et al.*, 2017 isolated toxigenic *C. difficile* from 5.5% to 11.3% of calves and cattle and considered them as persistent reservoirs that probably indirectly disseminate the pathogen to humans, *via* the environment. Rahimi *et al.*, 2014 evaluated meet of different animals for the presence of *C. difficile* and found the highest prevalence was in buffalo meat (9%).

Sheep and goats: Rates of infection with *C. difficile* in apparently healthy and diseased sheep were observed (14.29%), while in goats, no infection with *C. difficile* could be detected. Previous studies showed infection rates ranging from 0 to 18.2% (al Saif and Brazier, 1996; Rieu-Lesme and Fonty, 1999). Recent study reported *C. difficile* colonization in 4.2% to 11.2% of lambs and 0.6% of sheep, of which all *C. difficile* isolates from lambs were positive for tcdA and tcdB (A+, B+) but negative for binary toxin genes (CDT-), while the isolate from sheep was A- B+ CDT+ (Knight and Riley, 2013). Another recent study had detected *C. difficile* in 9.2% of neonatal goats, while none of the adult goats were positive (Avberšek *et al*., 2014). Romano *et al*., 2012 isolated *C. difficile* from 7.5% of goats irrespective to age.

Horses: The rate of infection with *C. difficile* in apparently healthy horses was 10%. Recent studies had considered *C. difficile* as one of the most significant causes of diarrhoea and enterocolitis in horses (Arroyo *et al*. 2006; Weese *et al*. 2006; Uzal *et al*. 2012; Diab *et al*. 2013b). Prevalence studies of *C. difficile* in horses with gastrointestinal disease recorded varied infection rates, ranging between 5 % and 63 %, while in apparently healthy horses, the reported prevalence varied between 0 and 29 % (Diab *et al.* 2013b). Båverud *et al*. 2003 reported up to 44 % colonization rate in apparently healthy foals under antibiotic treatment, which is considered the main risk factor leading to CDI in horses (Diab *et al*. 2013b). Similar infection rates (from 4.8 to 11 %) were observed in hospitalized horses without clinical signs of *C. difficile* disease (Rodriguez *et al.* 2013).

Dogs: In this study, the rate of infection with *C. difficile* in apparently healthy dogs was 14.29%. Struble et al., 1994 reported similar prevalence, in canines visited veterinary hospital in California, as they found that 13.8% of canines with normal stools shed *C. difficile*. Another study done by Weese *et al*. 2010b found that 10 % of dogs in households were colonized by *C. difficile*. Weese *et al.*, 2001 reported no infection rates (0%) with *C. difficile*, but *C. difficile* toxins A, B, or both were present in feces of 7% normal dogs. While, Lefebvre *et al.*, 2006 reported higher infection rates, as they isolated *C. difficile* from feces of 58% apparently healthy dog. Rodriguez-Palacios *et al*. 2013 noted that, within the same geographical area, all dog isolates resembled those found in human hospitals. Other studies (Schneeberg *et al*. 2012; Silva *et al*. 2013; Spigaglia *et al.* 2015) confirmed that dogs can be healthy carriers of human epidemic PCR-ribotypes *C. difficile* strains.

Cats: There were no infection with *C. difficile* reported in apparently healthy cats (0%). While, the rate of infection with *C. difficile* in diseased cats was 23.53%. These results agree with Madewell *et al.*, 1999 who could not detect *C. difficile* in healthy adult cats, but isolated *C. difficile* from 9.4% of diseased ones, and identified A and B toxins sequences in 34.8% of that infected group. Wei *et al*., 2019 isolated *C. difficile* from 7% of apparently healthy cats.

Our results showed that the toxigenic *C. difficile* is a common member of the fecal flora of companion and farm animals, which indicate that those animals are reservoirs and sources of pathogenic *C. difficile*, and that household, nosocomial and contact transmission of *C. difficile* from animals to humans cannot be denied. Moreover, as *C. difficile* is a spore former, it can survive for unspecified period in the environment and could be ingested by man via various routes.

The presence of *C. difficile* in both apparently healthy and diseased animals of different species suggests that contamination may affect the external environment and may play an important role in the expansion of pathogenic *C. difficile* and also in transmission to humans via food.

REFERENCES

Al Saif N and J Brazier, 1996. The distribution of Clostridium difficile in the environment of South Wales. J Med Microbiol, 45:133–137.

Álvarez-Pérez S, JL Blanco, T Pelaez, MP Lanzarot, C Harmanus, E Kuijper, *et al*., 2015. Faecal shedding of antimicrobial-resistant *Clostridium difficile* strains by dogs. J Small Anim Pract, 56(3):190–5.

Arroyo LG, HR Sta¨mpfli and JS Weese, 2006. Potential role of Clostridium difficile as a cause of duodenitisproximal jejunitis in horses. J Med Microbiol, 55:605–608.

Avbersek J, S Janezic, M Pate, M Rupnik, V Zidaric, K Logar, M Vengust, M Zemljic, T Pirs and M Ocepek, 2009. Diversity of *Clostridium difficile* in pigs and other animals in Slovenia. Anaerobe, 15:252–255.

Avberšek J, T Pirš, M Pate, M Rupnik and M Ocepek, 2014. Clostridium difficile in goats and sheep in Slovenia: Characterisation of strains and evidence of age-related shedding. Anaerobe, 28: 163-167.

Båverud V, A Gustafsson, A Franklin, A Aspán and A Gunnarsson, 2003. Clostridium difficile: prevalence in horses and environment, and antimicrobial susceptibility. Equine Veterinary Journal, 35: 465-471.

Clark T and M Wiselka, 2008. Clostridium difficile infection. Clin Med, 8: 544-547.

CDCP (Center for Disease Control and Prevention), 2005. Severe *Clostridium difficile*-associated disease in populations previously at low risk-four states, 2005. MMWR Morb Mortal Wkly Rep, 54:1201–1205.

Centers for Disease Control and Prevention, 2008. Surveillance for community-associated Clostridium difficile—Connecticut, 2006. MMWR Morb Mortal Wkly Rep, 57:340–343.

Dahms C, NO Hübner, F Wilke and A Kramer, 2014. Mini-review: Epidemiology and zoonotic potential of multiresistant bacteria and Clostridium difficile in livestock and food. GMS hygiene and infection control, 9(3): Doc21.

Diab SS, G Songer and FA Uzal, 2013.Clostridium difficile infection in horses: a review. Vet Microbiol, 167:42–49.

Heinlen L and JD Ballard, 2010. Clostridium difficile infection. Am J Med Sci, 340: 247-252.

Hoover DG, A Rodriguez-Palacios, 2013.Transmission of Clostridium difficile in foods. Infect Dis Clin North Am, 27:675–685.

Indra A, H Lassnig, N Baliko, P Much, A Fiedler, S Huhulescu and F Allerberger, 2009. *Clostridium difficile*: a new zoonotic agent?. Wien Klin Wochenschr, 121: 91-95.

Juneau C, EN Mendias, N Wagal, M Loeffelholz, T Savidge, S Croisant and S Dann, 2013. Community-acquired *Clostridium difficile* infection: awareness and clinical implications. J Nurse Pract, 9:1–6.

Koene MGJ, D Mevius, JA Wagenaar, C Harmanus, MPM Hensgens, AM Meetsma, FF Putirulan, MAP Van Bergen and EJ Kuijper, 2012. *Clostridium diff*icile in Dutch animals: their presence, characteristics and similarities with human isolates. Clin Microbiol Infect, 18:778–784.

Knight DR and TV Riley, 2013. Prevalence of Gastrointestinal Clostridium difficile Carriage in Australian Sheep and Lambs. Applied and Environmental Microbiology, 79 (18): 5689–5692.

Kuntz JL, EA Chrischilles, JF Pendergast, LA Herwaldt and PM Polgreen, 2011. Incidence of and risk factors for community-associated *Clostridium difficile* infection: a nested case-control study. BMC Infect Dis, 11: 194-10

Lefebvre SL, D Waltner-Toews, AS Peregrine, R Reid-Smith, L Hodge, LG Arroyo *et al*., 2006. Prevalence of zoonotic agents in dogs visiting hospitalized people in Ontario: implications for infection control. J Hosp Infect, 62(4):458-66.

Madewell BR, KJ Bea, SA Kraegel, M Winthrop, YJ Tang and J Silva, 1999. Clostridium difficile: a survey of fecal carriage in cats in a veterinary medical teaching hospital. J Vet Diagn Invest, 11:50–54

McFarland LV, 2008. Update on the changing epidemiology of Clostridium difficile-associated disease. Nat Clin Pract Gastroenterol Hepatol, 5:40–48.

Rabold D, W Espelage, M Abu Sin, T Eckmanns, A Schneeberg, H Neubauer *et al*., 2018. The zoonotic potential of Clostridium difficile from small companion animals and their owners. PLoS ONE, 13(2): e0193411.

Rahimi E, M Jalali and JS Weese, 2014. Prevalence of Clostridium difficile in raw beef, cow, sheep, goat, camel and buffalo meat in Iran. BMC Public Health, 14:119.

Rieu-Lesme F and G Fonty, 1999. Isolation of Clostridium difficile from the ruminal reservoir of newborn lambs. Vet Rec, 145:501.

Rodriguez C, B Taminiau, J Van Broeck, M Delme´ e and G. Daube, 2016. Clostridium difficile in Food and Animals: A Comprehensive Review. Advances in experimental medicine and biology, 932: 65-92.

Rodriguez C, DE Hakimi, R Vanleyssem, B Taminiau, J Van Broeck, M Delmee, N Korsak and G Daube, 2017. Clostridium difficile in beef cattle farms, farmers and their environment: assessing the spread of the bacterium. Vet Microbiol, 210:183–7.

Rodriguez-Palacios A, S Borgmann, TR Kline *et al*., 2013. Clostridium difficile in foods and animals: history and measures to reduce exposure. Anim Health Res Rev, 14:11–29.

Romano V, Albanese, F Dumontet, S Krovacek, K Petrini, and V Pasquale, 2012. Prevalence and Genotypic Characterization of Clostridium difficile From Ruminants in Switzerland. Zoonoses and Public Health, 59: 545–548.

Rupnik M, 2007. Is Clostridium difficile-associated infection a potentially zoonotic and foodborne disease?.Clin Microbiol Infect, 13:457–459.

Schneeberg A, M Rupnik, H Neubauer *et al*., 2012. Prevalence and distribution of Clostridium difficile PCR ribotypes in cats and dogs from animal shelters in Thuringia, Germany. Anaerobe, 18:484–488.

Silva ROS, RLR Santos, PS Pires *et al*., 2013. Detection of toxins A/B and isolation of Clostridium difficile and Clostridium perfringens from dogs in Minas Gerais, Brazil. Braz J Microbiol, 44:133–137

Songer JG, *et al*., 2009. Clostridium difficile in retail meat products, U.S.A., 2007. Emerg Infect Dis,15:819–821.

Spigaglia P, I Drigo I, F Barbanti *et al*., 2015. Antibiotic resistance patterns and PCR-ribotyping of Clostridium difficile strains isolated from swine and dogs in Italy. Anaerobe, 31:42–4.

Struble AL, YJ Tang, PH Kass *et al*., 1994. Faecal shedding of Clostridium difficile in dogs: a period prevalence survey in a veterinary medical teaching hospital. J Vet Diagn Invest, 6: 342—347.

Uzal FA, SS Diab, P Blanchard *et al*., 2012. Clostridium perfringens type C and Clostridium difficile co-infection in foals. Vet Microbiol, 156:395–402.

Warny M, J Pepin, A Fang, G Killgore, A Thompson, J Brazier *et al*., 2005. Toxin production by an emerging strain of *Clostridium difficile* associated with outbreaks of severe disease in North America and Europe. Lancet, 366: 1079-108

Weese JS, HR Staempfli, JF Prescott, SA Kruth, SJ Greenwood and HE Weese, 2001. The roles of Clostridium difficile and enterotoxigenic Clostridium perfringens in diarrhea in dogs. J Vet Intern Med, 15 (4):374-8.

Weese JS, L Toxopeus and L Arroyo, 2006. Clostridium difficile associated diarrhoea in horses within the community: predictors, clinical presentation and outcome. Equine Vet J, 38:185–188.

Weese JS, T Wakeford, R Reid-Smith, J Rousseau and R Friendship, 2010a. Longitudinal investigation of *Clostridium difficile* shedding in piglets. Anaerobe, 16:501–504.

Weese JS, R Finley, RR Reid-Smith *et al*. 2010b.Evaluation of Clostridium difficile in dogs and the household environment. Epidemiol Infect 138: 1100–1114.

Wei Y, M Sun, Y Zhang, J Gao, F Kong, D Liu, H Yu, J Du and R Tang, 2019. Prevalence, genotype and antimicrobial resistance of Clostridium difficile isolates from healthy pets in Eastern China. BMC Infectious Diseases, 19:46.

Wetterwik KJ, G Trowald-Wigh, LL Fernstrom and K Krovacek, 2013. *Clostridium difficile* in faeces from healthy dogs and dogs with diarrhea. Acta Vet Scand, 55:23.

Zilberberg MD, AF Shorr and MH Kollef, 2008. Increase in adult *Clostridium difficile*-related hospitalizations and case-fatality rate, United States, 2000-2005. Emerg Infec Dis, 14: 829-831.