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Natasza M. R. Hain-Saunders

Daniel R. Knight

Mieghan Bruce

Thomas V. Riley
*Edith Cowan University*, t.riley@ecu.edu.au



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Minireview

# *Clostridioides difficile* infection and One Health: an equine perspective

**Natasza M.R. Hain-Saunders**,[1] **Daniel R. Knight**,[1,2] **Mieghan Bruce**[3] **and Thomas V. Riley**[1,2,4,5*]

[1]*Biosecurity and One Health Research Centre, Harry Butler Institute, Murdoch University, Murdoch, WA, Australia.*

[2]*School of Biomedical Sciences, The University of Western Australia, Queen Elizabeth II Medical Centre, Nedlands, WA, 6009, Australia.*

[3]*School of Veterinary Medicine, Centre for Biosecurity and One Health, Murdoch University, Murdoch, WA, Australia.*

[4]*School of Medical and Health Sciences, Edith Cowan University, Joondalup, WA, Australia.*

[5]*Department of Microbiology, PathWest Laboratory Medicine, Queen Elizabeth II Medical Centre, Nedlands, WA, Australia.*

## Summary

***Clostridioides (Clostridium) difficile*** **presents a significant health risk to humans and animals. The complexity of the bacterial–host interaction affecting pathogenesis and disease development creates an ongoing challenge for epidemiological studies, control strategies and prevention planning. The recent emergence of human disease caused by strains of *C. difficile* found in animals adds to mounting evidence that *C. difficile* infection (CDI) may be a zoonosis. In equine populations, *C. difficile* is a known cause of diarrhoea and gastrointestinal inflammation, with considerable mortality and morbidity. This has a significant impact on both the well-being of the animal and, in the case of performance and production animals, it may have an adverse economic impact on relevant industries. While *C. difficile* is regularly isolated from horses, many questions remain regarding the impact of asymptomatic carriage as well as optimization of diagnosis, testing and treatment. This review provides an overview of our understanding of equine CDI while also identifying knowledge gaps and the need for a holistic One Health approach to a complicated issue.**

## Introduction

First isolated in 1935 from the intestinal flora of human infants, *Clostridioides (Clostridium) difficile* was initially considered a commensal (Hall and O'Toole, 1935). This perception remained for four decades until *C. difficile* was finally identified as a causative agent of antimicrobial-related diarrhoea and life-threatening pseudomembranous colitis (Bartlett *et al*., 1978; Larson *et al*., 1978). Today, *C. difficile* is recognized as a major cause of gastrointestinal disease affecting both animals and humans, with its ubiquity in the environment becoming increasingly apparent (Lim *et al*., 2020).

In human populations, *C. difficile* is the most common cause of infectious healthcare-associated diarrhoea with the rate of severe cases increasing (McDonald *et al*., 2018). In the last 20 years, however, focus has turned to the role of *C. difficile* in animal gastrointestinal disease and the role of animal populations in the amplification and transmission of *C. difficile*. High prevalence of *C. difficile* has been consistently reported globally in both swine (mean 43%, range 0%–100%) and cattle (mean 14%, range 0.5%–56.4%), with a 2014 study confirming the relatedness of strains isolated from paired pig and farmer samples (Knetsch *et al*., 2014; Knight and Riley, 2019). Further genomic studies have provided compelling evidence for a novel zoonotic paradigm for *C. difficile* infection (CDI) (Knight *et al*., 2017; Knight *et al*., 2019). A 2017 study strengthened this animal–human link, identifying a significant association between proximity to livestock farms and the occurrence of community-acquired CDI case clusters (Anderson *et al*., 2017). These key aspects have led to a deeper consideration of the impact of *C. difficile* in a wider range of animal populations.

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In horses there are three inter-related issues pertaining to *C. difficile*. Primarily there is an animal welfare concern; disease in horses due to *C. difficile* can cause discomfort in its mildest form and debilitating complications and death at its most severe. Horses are, however, also intertwined with human activity; used as performance, work and companion animals. In 2018/19, the horse racing industry in Australia reportedly contributed AU$9.3 billion to the Australian GDP (Racing Australia, 2020) The socio-economic impact of CDI on racing, breeding and other equine-related industries is therefore potentially of great significance. Finally, in the One Health era, which acknowledges the link between human health, animal health and the environment, the role of horses in the dissemination and dispersal of *C. difficile* to the wider community and environment needs to be considered. The review outlines our current understanding of *C. difficile* and CDI within equine populations. It reflects on the knowledge gaps and diagnostic shortfalls evident within this emerging field and the importance of adopting a One Health approach to achieve effective infection prevention and control and improved health outcomes for humans and animals alike.

## Pathophysiology

The pathophysiology of CDI is similar in horses compared to humans and other animals. CDI refers to the colonization of *C. difficile* within the host tissue. Disease associated with CDI is toxin-mediated and exhibits a broad spectrum of signs and symptoms. Mild cases manifest as watery diarrhoea and low-grade fever. Further infection development may result in a progression to severe CDI, with additional features of haemodynamic instability, pseudomembranous colitis and severe anorexia (Bartlett *et al*., 1978). In horses, *C. difficile* is also a known cause of duodenitis-proximal jejunitis and necrotizing enteritis (Arroyo *et al*., 2004; Arroyo *et al*., 2017). Extracolonic manifestations such as bacteremia and organ failure can also develop and extreme cases can result in death (Dallal *et al*., 2002; Arroyo *et al*., 2004; Napolitano and Edmiston, 2017).

Transmission of *C. difficile* occurs through the faecal–oral route. Ingested spores pass to the bowel where bile acids stimulate germination into vegetative cells (Francis *et al*., 2013). These cells proliferate in the intestinal anaerobic environment, penetrating the mucus layer to attach to the host epithelial cells. Following attachment, toxigenic strains produce toxins that interfere with cell signalling, disrupting the cytoskeleton resulting in cell damage, loss of tight junction integrity and apoptosis (Hecht *et al*., 1992). This damage induces inflammatory mediator release and fluid secretion which manifests as watery diarrhoea (Pruitt and Lacy, 2012).

Colonization and lesion development associated with host inflammatory response to CDI occurs within the intestinal tract; however, the exact location varies between animal species and stage of life (Keel and Songer, 2006). In neonatal foals (≤1 month old), lesions are predominantly located within the small intestine with extended formation within the large intestine less frequent (Keel and Songer, 2006; Diab *et al*., 2013b). Conversely, lesion development in older foals and adult horses appears to be restricted to the cecum and ascending colon of the large intestine (Keel and Songer, 2006).

Clinical manifestations may be self-resolving or chronic. Despite recurrent CDI occurring in 20%–30% of human *C. difficile* cases, recurrent CDI has not been noted as an ongoing issue in equine populations (Weese *et al*., 2006; Cornely *et al*., 2012). A lack of long-term surveillance of *C. difficile* and CDI in horses, however, may be impacting this view. Asymptomatic infection can also occur resulting in the shedding of viable spores in the absence of disease, contributing to contamination of the environment (Båverud *et al*., 2003). This complexity creates difficulty in discerning between states of carriage, colonization and infection. Further investigation into this complexity and the disparity of disease impact within and between species may indeed lead to further understanding of *C. difficile* pathophysiological nuances (Weese, 2020).

## Pathogenicity

The pathogenicity of *C. difficile* is attributed to the production of potent toxins as well as the ability to form hardy endospores, and these characteristics may appear in human, animal and environmental strains alike. Toxigenicity is influenced by the presence of the Pathogenicity Locus (PaLoc) – a 19.6 kb chromosomal region that encodes toxin A (*tcdA*) and toxin B (*tcdB*), as well as positive and negative regulators for toxin expression (*tcdR* and *tcdC* respectively) (Braun *et al*., 1996; Knight *et al*., 2015b). The presence of an additional binary toxin (*C. difficile* transferase, CDT), thought to enhance pathogenicity, has also become increasingly significant in the last two decades. CDT appears to be highly prevalent in animal strains (Knight *et al*., 2013; Gerding *et al*., 2014; Knight *et al*., 2015a). The genetic architecture of the *C. difficile* PaLoc and binary toxin locus (CdtLoc) is shown in Fig. 1. While toxigenic strains of *C. difficile* are undoubtedly important due to their association with symptomatic disease, it has been demonstrated experimentally that acquisition of the *C. difficile* PaLoc region by non-toxigenic strains can occur via horizontal gene transfer (HGT), although the frequency at which this occurs is not known (Brouwer *et al*., 2013; Elliott *et al*., 2014; Candel-

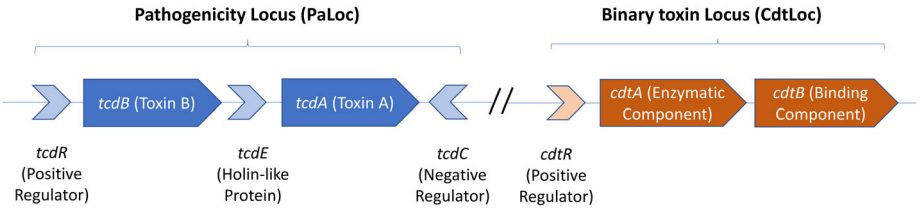


**Fig. 1.** Schematic diagram of the *C. difficile* PaLoc and CdtLoc chromosomal regions which encode for virulence factors, toxin A and toxin B, and binary toxin respectively. Adapted from Francis *et al*. (2013) and Elliott *et al*. (2017).

Pérez *et al*., 2019). Recombination and HGT are thought to have played a significant role in the evolution of 'hyper-virulent' *C. difficile* strains seen today, including PCR ribotype (RT) 027 which caused human CDI epidemics in Canada, the USA and Europe and has been isolated from horses (Songer *et al*., 2009; He *et al*., 2013). Genomic studies have determined that approximately 11% of the *C. difficile* genome is comprised of mobile genetic elements including transposons and plasmids carrying antimicrobial resistance (AMR) genes (Sebaihia *et al*., 2006).

Non-toxigenic strains of *C. difficile* are also thought to have a protective function against toxigenic strains (Natarajan *et al*., 2013). Although this is yet to be investigated in equine populations, protection has been seen experimentally in pigs (Songer *et al*., 2007; Oliveira Júnior *et al*., 2019). This attribute could potentially be exploited in the production of preventative medications or vaccines, as seen with the non-toxigenic *C. difficile* human strain, NTCD-M3, which is showing promising results in phase II human trials for the prevention of CDI (Gerding *et al*., 2015; Zhang *et al*., 2015; Gerding *et al*., 2018). For this reason, surveillance of both toxigenic and non-toxigenic strains in healthy and diseased hosts is critical in understanding the aetiology and epidemiology of CDI, and for the early detection of emerging strains.

Another factor contributing to *C. difficile* pathogenicity is the ability to form hardy endospores following exposure to stress (Kochan *et al*., 2018). As an obligate anaerobe, the formation of spores allows survival outside the host and the versatility to persist in diverse environments. Inoculated horse faeces can harbour viable *C. difficile* for 4 years despite being exposed to the natural environment (Båverud *et al*., 2003). These *C. difficile* spores can also withstand extreme temperatures and are impervious to conventional chemicals including alcohol-based sanitizers commonly used in infection prevention and control (Fawley *et al*., 2007; Hellickson and Owens, 2008). This highlights the durability of *C. difficile* in both human and animal settings and is a cause for major concerns for public health, and agricultural and animal husbandry practices. Despite 40 years of investigations, the infectious dose of *C. difficile* in humans and animals is not known, although murine models suggest that this could be as low as 1 spore $cm^{-2}$ in healthy mice (Lawley *et al*., 2010). The process is further complicated by the need for microbiota disruption prior to exposure (Moono *et al*., 2016). It is therefore important to maximize the detection of even small numbers of *C. difficile* spores present within samples until further investigation into infectious dose.

## Epidemiology

The earliest record of *C. difficile* in equines was in 1984 (Ehrich *et al*., 1984); however, the first suggestion of an association with equine enterocolitis was proposed 3 years later following an outbreak in a group of diarrhetic foals (Jones *et al*., 1987). To date, there have been inconsistencies in the reported prevalence and perceived impact of *C. difficile* in horses (Diab *et al*., 2013a). Isolation of *C. difficile* has long been associated with horses with diarrhoea or acute colitis, with isolation rates ranging from 5% to 90% (Båverud *et al*., 2003; Frederick *et al*., 2009; Thean *et al*., 2011; Morsi *et al*., 2019).

The proportion of healthy adult horses that carry *C. difficile* appears to be much lower. Earlier small-scale investigations of *C. difficile* in the Northern hemisphere returned relatively low detection rates (0%–4%), (Madewell *et al*., 1995; Weese *et al*., 2001; Båverud *et al*., 2003), while a single preliminary Australian study failed to isolate *C. difficile* from healthy horses ($n = 112$) (Thean *et al*., 2011) This is in contrast to a larger study in Ontario in 2011 which returned an overall faecal prevalence of 7.6% in healthy adult racehorses ($n = 540$) and, more recently, smaller studies in Minnesota, USA ($n = 50$) and Italy ($n = 24$) which recorded a 14% and 25% prevalence of *C. difficile* respectively (Ossiprandi *et al*., 2010; Medina-Torres *et al*., 2011; Shaughnessy *et al*., 2018). Table 1 summarizes the prevalence of *C. difficile* identified in these key studies.

The prevalence of *C. difficile* appears higher in foals, with younger animals tending to harbour the bacterium at higher rates, similar to other young animals (Båverud *et al*., 2003; Morsi *et al*., 2019). In a 2003 study in Sweden, *C. difficile* was isolated from 29% of healthy foals under the age of 14 days, and only 0.6% of foals aged greater than 14 days (Båverud *et al*., 2003). This

**Table 1.** Summary of key studies on the prevalence of *C. difficile* in horses.

| Location | Year(s) | Number tested | Health Status | Adult/ Foal | No. *C. difficile* positive (%) | References |
|---|---|---|---|---|---|---|
| Australia | 2007–2009 | 62 | Diarrhetic | Unknown | 14 (23%) | Thean *et al*. (2011) |
| | | 112 | Healthy | Unknown | 0 (0%) | |
| Canada (Ontario) | 1998–1999 | 55 | Diarrhetic | Adult | 7 (12.7%) | Weese *et al*. (2001) |
| | | 255 | Healthy | Adult | 1 (0.4%) | |
| | | 31 | Diarrhetic | Foal | 11 (33.3%) | |
| | | 47 | Healthy | Foal | 0 | |
| Canada (Ontario) | 2006–2008 | 540 | Healthy | Adult | 41 (7.59%) | Medina-Torres *et al*. (2011) |
| Italy | 2007 | 24 | Healthy | Adult | 6 (25%) | Ossiprandi *et al*. (2010) |
| | | 18 | Healthy | Foal | 8 (44.4%) | |
| Saudi Arabia | 2019 | 30 | Diarrhetic | Adult | 7 (23.3%) | Morsi *et al*. (2019) |
| | | 286 | Healthy | Adult | 3 (1.1%) | |
| | | 49 | Diarrhetic | Foal | 11 (22.5%) | |
| | | 42 | Healthy | Foal | 3 (7.1%) | |
| Sweden | Unknown | 227 | Enteric disorders/on antimicrobials | Adult | 23 (10.1%) | Båverud *et al*. (2003) |
| | | 273 | Healthy | Adult | 0 | |
| | | 51 | Enteric disorders/on antimicrobials | Foal | 11 (21.57%) | |
| | | 226 | Healthy | Foal | 17 (8%) | |
| USA (California) | 1993 | 10 | Diarrhetic | Adult | 9 (90%)[a] | Madewell *et al*. (1995) |
| | | 23 | Healthy | Adult | 1 (4.3%) | |
| USA (Minnesota) | 2011–2013 | 50 | Healthy | Unknown | 7 (14%) | Shaughnessy *et al*. (2018) |
| USA (Florida) | 2003–2008 | 233 | Diarrhetic | Foal | 11 (5%) | Frederick *et al*. (2009) |

[a]Possible hospital outbreak.

trend was also identified in a 2019 study in Saudi Arabia where all foals carrying *C. difficile* (7.1% of healthy and 22.5% of diarrhetic foals) were aged <2 months, with *C. difficile* not isolated from any foal over this age (Morsi *et al*., 2019).

It is difficult to draw meaningful conclusions from these variable results given the limited number of investigations, combined with geographical, methodological and temporal differences. Nevertheless, there does appear to be a tendency towards outbreaks and sporadic cases rather than ongoing chronic or recurrent illness (Diab *et al*., 2013b). Longitudinal studies further revealed the transient nature of horse *C. difficile* colonization, with an overall prevalence of 5.4% compared to a cumulative prevalence of 40% (Schoster *et al*., 2012). This was concordant with a recent Swiss study investigating *C. difficile* in horses with colic, and diarrhetic and healthy horses where the cumulative prevalence (19%) appeared much higher than single-day testing (10%), questioning the need for multi-day sampling in at-risk horses or suspected cases (Schoster *et al*., 2019).

This ephemeral pattern has been demonstrated in other animals and adds to the complexity of CDI epidemiology and difficulty in comparing studies (Bandelj *et al*., 2016). Furthermore, the opportunistic nature of *C. difficile* colonization and the need for both exposure and commensal flora disruption for the establishment of disease creates challenges in determining the significance of an asymptomatic state. Despite these apparent inconsistencies and knowledge gaps, it is evident that *C. difficile* in horses could potentially act as a reservoir for zoonotic spread and further investigation is needed to clarify this role.

Equine *C. difficile* strains isolated in studies include both novel strains as well as those identified in other animals, the environment and humans. Notably, in the 2011 Ontario study mentioned 76.5% of *C. difficile* isolated were strains previously isolated in humans locally, with 57.7% being RTs 001, 027 or 078, which have been implicated internationally in epidemic outbreaks in humans and other animals (Medina-Torres *et al*., 2011). Concerningly, equine cases of infection with the highly virulent RT 027 strain have also been identified elsewhere with severe outcomes (Songer *et al*., 2009).

## Predisposing factors for CDI

Risk factors for CDI in horses centre around circumstances that disrupt the host's native intestinal flora, or which create situations of higher exposure. Antimicrobial exposure and hospitalization are the most recognized risks and have long been associated with CDI across human and animal populations alike (Deshpande *et al*., 2013; Slimings and Riley, 2014).

### *Antimicrobial use*

Antimicrobials contribute to disease by altering the number, diversity and relative composition of the host commensal gut flora, allowing *C. difficile* to colonize

(Robinson and Young, 2010; Reeves *et al*., 2011). Studies suggest certain antimicrobials may also increase adhesin (Denève *et al*., 2008) and toxin gene (Drummond *et al*., 2003) expression in *C. difficile*, leading to increased pathogenicity.

CDI in horses has been associated with exposure to an array of antimicrobials including $\beta$-lactams (penicillin, ampicillin, cephalosporins), gentamicin, clindamycin, erythromycin, rifampicin and trimethoprim/sulfonamides (Båverud *et al*., 1997; Båverud *et al*., 1998; Arroyo *et al*., 2004; Diab *et al*., 2013a; Morsi *et al*., 2019). Of particular note is the association of CDI with the use of ceftiofur, one of the antimicrobials most commonly used in horses (Rodriguez *et al*., 2014). Ceftiofur is a veterinary third-generation cephalosporin, the human equivalent of which is also a known risk factor for CDI in humans (Slimings and Riley, 2014). Ceftiofur can significantly disrupt the bacterial flora of the horse hindgut with studies identifying a 75% reduction in lactobacilli and the appearance of *C. difficile* within 24 h of antimicrobial administration (Harlow *et al*., 2013). This imbalance can allow opportunists such as *C. difficile* to colonize. Commensal bacteria in the horse gut are important for understanding *C. difficile* colonization for several reasons. First, it is believed that these bacteria compete for both nutrients and adhesion sites. Second, studies have also suggested that species such as lactobacilli alter their environment, producing metabolites utilized by certain bacteria and excluding others (Harlow *et al*., 2013). Commensal bacterial counts remain disrupted for at least 1 week after antimicrobial administration (Harlow *et al*., 2013). This is an important consideration as longer disruption increases the chance of exposure to pathogens such as *C. difficile* while in a high-risk state.

In addition to direct administration, indirect exposure to antimicrobials may also be important. Although not studied in horses, it has been estimated that between 15% and 50% of antimicrobials administered to livestock remain as residue in resulting manure with some thought to persist for over a year (Chee-Sanford *et al*., 2009; Kim *et al*., 2011; Berendsen *et al*., 2018; Filippitzi *et al*., 2019). Tetracyclines, macrolides, quinolones and lincosamide appeared to have the longest persistence in manure and the environment (Berendsen *et al*., 2018). Interestingly, the latter class includes clindamycin which has been linked to a greater risk of community-associated CDI (CA-CDI) development in humans (Deshpande *et al*., 2013). Studies in horses have also shown that mares of macrolide-treated foals have contracted CDI and hyperacute colitis due to the ingestion of residual antimicrobials, and outbreaks of colitis on horse farms due to feed contamination by tetracyclines have also been documented (Båverud *et al*., 1998; Keir *et al*., 1999).

*Hospitalization*

While hospitalization is generally accepted as a risk factor for *C. difficile* and CDI in horses, the primary source of exposure remains less clear. Environmental sampling at veterinary hospitals identified rough, hard to clean surfaces (such as concrete and mats), high traffic zones, and areas previously used by individuals with confirmed CDI as high-risk areas for transmission (Weese *et al*., 2000). However, in a recent study, nosocomial equine CDI was presented as an increasingly complex and multifaceted issue (Weese *et al*., 2021). The nature and severity of illness at admission, undefined classification of 'hospital' versus 'community' acquired cases in an equine setting and extent of contact with treating veterinarians all add to the overall narrative and must be considered in the identification of preventative strategies and infection control protocols.

*Other factors*

Diet changes, transportation and other causes of stress in animals may act as risk factors for CDI in equine populations (Båverud, 2002). Such influences have been previously identified in cattle and are thought to disrupt the gut flora, providing a window of opportunity for *C. difficile* to establish; however, the exact mechanisms and the full impact are not known (Bandelj *et al*., 2016). Despite this extensive list, it should be noted, however, that cases of CDI with no obvious risk factors are common. This is particularly true in foals which may become colonized within days of birth, but also in a proportion of adult equine cases (Båverud *et al*., 2003). This wide array of potential predisposing factors and uncertainty shows the complexity of CDI and highlight the challenges faced in controlling its impact.

## Presentation, detection and diagnosis

Equine CDI can have a rapid onset, with a delay in treatment leading to significant patient deterioration. With reported mortality of up to 83% in confirmed CDI cases (Nomura *et al*., 2020), a need for timely investigation and diagnosis based on a combination of clinical history, presentation and laboratory testing is apparent.

As with humans, the clinical presentation of CDI in horses can vary in both clinical signs and severity. Horses with CDI may exhibit episodes of watery diarrhoea, abdominal distension, fever, tachypnoea, tachycardia, changes to the mucous membranes and capillary refill times, as well as depression and anorexia (Weese *et al*., 2006). Intestinal inflammation and lesion development are common in both foals and adult horses, with the region thickened due to oedema, and characterized by

haemorrhage, eruption and necrosis of the mucosa, and pseudomembrane formation (Keel and Songer, 2006; Diab *et al*., 2013b). These clinical signs and symptoms, however, are common to a variety of aetiologies and are insufficient indicators alone for a presumptive diagnosis of CDI. Differential diagnoses cover a diverse selection of infectious agents such as *Salmonella* species, Equine Coronavirus, *Neorickettsia risticii* and *Clostridium perfringens* (Shaw and Stämpfli, 2018). Laboratory identification, therefore, plays an important role in diagnosis, although this is not without its problems.

Standardized testing protocols do not exist across veterinary laboratories (Medina-Torres *et al*., 2010). Despite progression in technology and laboratory systems in the last few decades, the most optimal method for detection of *C*. *difficile* and subsequent diagnosis of CDI remains a contentious issue across veterinary and human medical fields (Fang *et al*., 2017). As with all clinical testing, a delicate balance must be struck between sensitivity and specificity as well as efficiency and cost. In the case of *C*. *difficile*, however, the complexities of the pathogenesis of CDI, combined with the phenomenon of asymptomatic carriage, create a further obstacle, and the lines between detection and diagnosis begin to blur. There are currently three main laboratory testing methods utilized in the detection of *C*. *difficile* and diagnosis of CDI. These include culture, enzyme-linked immunosorbent assays and PCR. Ongoing research efforts into additional testing options have, however, shown potential.

### *Culture with cell cytotoxin assay*

Techniques involving culturing of *C*. *difficile* from faecal samples and testing isolates for toxin production are recognized as the gold standard for laboratory detection (Planche *et al*., 2013). Due to the supposed difficulty in culturing this bacterium (from which it gained its name), and the low vegetative cell/high spore count in animal faecal samples, various enrichment broths containing antimicrobials are often utilized in addition to direct culture (Knight *et al*., 2014; Avberšek, 2017). This is followed by sub-culture onto selective and differential media such as cycloserine-cefoxitin fructose agar or chromogenic agar (Avberšek, 2017).

Simple culturing *C*. *difficile* is insufficient to discriminate between toxin and non-toxin producing strains. Subsequent tests such as a cell culture cytotoxicity assay are therefore required to determine toxigenicity and, in turn, the capacity to cause disease. The long turnaround time for growth (24–48 h) and toxin assays deem this approach impractical for routine diagnostic use. Furthermore, *C*. *difficile* culturing procedures across laboratories are not standardized (Carroll, 2011). Culturing is therefore generally reserved for epidemiological investigations and as a reference method (McDonald *et al*., 2018).

### *Enzyme-linked immunosorbent assay*

Enzyme-linked immunosorbent assays (ELISAs) can detect glutamate dehydrogenase (GHD), a highly conserved enzyme produced by all *C*. *difficile* in faecal samples (Carman *et al*., 2012). This method is quick and inexpensive; however, it lacks specificity to distinguish between toxigenic and non-toxigenic strains. Several commercial EIA kits have been developed and are utilized in diagnostic laboratories. Assessment of the most commonly used kit for equine *C*. *difficile* in North America showed a sensitivity of 86% and specificity of 96%; however, this is likely variable across competing products (Medina-Torres *et al*., 2010). ELISA kits aimed at detecting the presence of toxins A and B in faecal samples (with or without GHD) have also been developed. Given the requirement for toxin production for disease development, as well as the ease and availability of ELISA kits, these are now often routinely used as the diagnostic standard despite lower sensitivity and with many lacking formal validation in equine settings (Ramos *et al*., 2020).

### *PCR*

PCR is being increasingly utilized in commercial laboratories as a quick and very sensitive method for the detection of *C*. *difficile*, despite greater expense compared to ELISA (Planche *et al*., 2013). This method detects the presence of *C*. *difficile* genes or its toxin genes within the sample. Caution must be employed for the diagnosis of disease as this method does not identify toxins, just toxin genes, and fails to distinguish between transient carrier and permanent colonization states (Oliver-Espinosa, 2018). For this reason, CDI overdiagnosis through the reliance on PCR testing alone has become a concern. In human studies, while negative predictive values remain high (96%), CDI positive predictive values can be as low as 46% and are highly dependent on disease prevalence (Lee *et al*., 2021). This highlights a need to better understand the extent of asymptomatic carriage within a population and the role it plays in CDI development and dissemination in parallel to decisions regarding diagnostic methods.

### *Future developments in diagnostics*

As knowledge of the bacterium and disease progresses, the possibility of additional diagnostic methods increases. For example, a recent study of blood biomarkers in

407 Arabian horses identified increased haptoglobin, serum amyloid A, neopterin and procalcitonin in horses with active *C. difficile* enterocolitis, as well as evidence of oxidative stress markers (El-Deeb *et al*., 2020). Although limited, this investigation into additional *C. difficile* markers shows the potential for future paths in CDI detection.

To overcome the shortfalls of current testing methodologies, a multistep testing regime may assist to increase the sensitivity and specificity of individual tests. Although specific recommendations in equine testing are yet to be made, the call for a two-step diagnostic method for animal *C. difficile* assays is repeatedly echoed throughout the literature. For example, researchers in the evaluation of pig testing recommended the successive use of both real-time PCR and toxigenic culture to overcome poor performance and inconsistency in EIA kits (Keessen *et al*., 2011), while Fathy *et al*. (2021) promote a combination of conventional culture followed by molecular methods to reduce false-negative results (Fathy *et al*., 2021).

There is, however, some concern regarding the methodologies currently utilized in equine *C. difficile* detection and CDI diagnosis. First and foremost is the frequent use of methods developed for human samples, but not yet validated for equine samples (Medina-Torres *et al*., 2010). This leaves many questions regarding the appropriateness of use and comparative performance in animal investigations and highlights the need for further analysis and species-specific testing.

Furthermore, limited understanding of the toxins identified in equine CDI may have an impact on laboratory diagnosis. While it is accepted that *C. difficile* toxins A and B are associated with cytopathic damage, the implications of the different combinations of toxins are not well known, creating issues in laboratory protocols that may focus only on the detection of a single toxin. In addition, the role of binary toxin in equine disease is not well understood and detection is not usually included in routine testing regimes. On a final note, as with all testing regimes, it is important that decisions on testing and diagnosis do not ignore practical issues such as whether the test outcome will have an impact on clinical decision making and alter the treatment strategy. As suggested by the international Equine Colitis Research Group, to make the most appropriate choice regarding how to test and, indeed, whether or not to test in the first place, the prevalence in healthy populations and the positive predictive value of the test must be known (International Equine Colitis Research Group, 2020). Perhaps more studies in a research setting utilizing the 'gold standard' method of toxigenic culture are required.

## Treatment and prophylaxis

On initial presentation, equine CDI cases with diarrhoea and endotoxemia associated with the disease can often represent an immediate danger that can lead to dehydration, electrolyte imbalances and haematological abnormalities (Weese *et al*., 2006; Nomura *et al*., 2020). Fluid and electrolyte therapy aimed at restoring blood volume and biochemistry is often carried out to stabilize the patient, with nonsteroidal anti-inflammatory agents administered to minimize deleterious inflammatory responses (Shaw and Stämpfli, 2018). Treatment to avoid complications associated with CDI is also important in the care of equine cases, including hoof cryotherapy to prevent laminitis (Shaw and Stämpfli, 2018).

These initial treatments, however, focus on correcting the effects of the infection rather than controlling the bacteria and toxins, themselves. In equine cases, a combination of antimicrobial and supportive therapies, therefore, remain central in the overall treatment of CDI. Metronidazole is often the first-line choice in the treatment of CDI in horses, with administration associated with survival (Weese *et al*., 2006). Concerningly, the existence of metronidazole resistance has been noted in some equine and human studies, highlighting the need for multiple avenues for treatment to be available (Boekhoud *et al*., 2020). Vancomycin may be utilized in cases where the infecting strain of *C. difficile* shows resistance to metronidazole; however, this should be avoided where possible due to the heavy reliance on vancomycin in human treatment and the rise of vancomycin resistance (Schoster and Staempfli, 2016). This thinking is being challenged with the increase in *C. difficile* resistance to metronidazole and a range of other antimicrobials over the last two decades (Peng *et al*., 2017). The AMR situation has become so dire that in both 2013 and 2019, the United States CDC listed *C. difficile* in the top five infectious agents posing an urgent threat to the community based on the apparent increase in AMR in circulating strains (Centers for Disease Control and Prevention, 2019).

Adjunctive therapies have also been developed with varying results. Bismuth subsalicylate is thought to prevent attachment of *C. difficile* to intestinal cells by coating the mucosa as well as providing antimicrobial and anti-inflammatory activity against *C. difficile* (Mallicote *et al*., 2012; Pitz *et al*., 2015). Despite its common use in diarrhetic horses, its true effectiveness in horse infections of the large intestine has been questioned due to the large volume of contents with little species-specific testing (McConnico, 2015). Di-tri-octahedral smectite also binds and neutralizes *C. difficile* toxins A and B *in vitro*, however, while commercial products (such as Bio-Sponge) are successfully utilized in the general treatment of diarrhetic horses, *C. difficile* specific *in vivo* testing is lacking (Weese *et al*., 2003; Hassel *et al*., 2009; Oliver-Espinosa, 2018).

Beyond traditional treatment methods, alternative microbiota restorative therapies are also being developed

aimed at re-establishing commensal microbiota diversity to resemble that of a 'healthy' individual. Faecal microbial transplantation (FMT) transfers faecal matter from healthy donors into the gastrointestinal tracts of CDI affected patients (Kelly *et al*., 2015). This has recently gained popularity in the treatment of recurrent human CDI with cure rates of 87%–90% (Kelly *et al*., 2015; van Beurden *et al*., 2017). The concept of FMT is not new in the animal setting. In its most basic form coprophagia, where one individual consumes the faeces of another, is commonplace between foals and their dams as an important process in establishing 'normal' gut bacteria during infancy (Quercia *et al*., 2019). The effects of this on *C. difficile* and CDI have not been exclusively investigated.

Transfaunation, as FMT is also known in animals, is well established in the treatment of general gastrointestinal ailments in livestock, including horses, although primarily anecdotal data exists for the latter (Feary and Hassel, 2006; Bakken, 2009). In recent years, better studies into the benefits of FMT in horses have emerged with promising results (McKinney *et al*., 2021). While large-scale studies in horses and other animals have not been done, isolated cases of treatment in marmosets and dogs have been largely successful (Yamazaki *et al*., 2017; Sugita *et al*., 2019). It is clear that much more needs to be done to enable FMT to become a mainstream treatment option in horses. A meeting of the International Equine Colitis Research Group in 2020 cited a lack of robust clinical studies into FMT in horses as a limiting factor in progressing this therapy, advising that many questions remain concerning longevity, screening and best practice protocols in horses (International Equine Colitis Research Group, 2020). The group also highlighted a gap in knowledge regarding the horse microbiome as a whole and a need for targeted investigations into key characteristics of horses affected by infectious agents such as *C. difficile*.

Preventative therapies may also play an important role in minimizing the effects of CDI in the future; however, to date success has been limited. Probiotics have generated some interest although with varied and inconsistent results (Schoster *et al*., 2015). Schoster *et al*. (2014) suggested that this inconsistency may have been a result of strain and dosage selection with some questions surrounding the quality control of commercial products. Despite this, a small number of specific probiotic agents have shown promising results. *Lactobacillus reuteri* reduces the adhesion of *C. difficile* to epithelial cells and significantly reduces the number of clostridial cells in the faeces of horses (Dicks *et al*., 2015). Similarly, *Saccharomyces boulardii* has also shown potential in the prevention of equine CDI following success in humans (Desrochers *et al*., 2005; Boyle *et al*., 2013; Carstensen *et al*., 2018). This microorganism releases proteases that digest *C. difficile* toxin A, reduce its ability to bind to host intestinal cells and interfere with host cell signalling to reduce damaging inflammatory responses (Castagliuolo *et al*., 1996; Chen *et al*., 2006). Vaccines for animals or humans are yet to be developed although a number have progressed to phases II and III trials (Riley *et al*., 2019). It is clear that further investigations into prevention and alternative treatments for CDI in horses are required.

## A changing landscape and call for a One Health approach

Although traditionally considered a healthcare-related disease, cases of CA-CDI are becoming increasingly common, now accounting for up to 50% of all human CDI cases (Ofori *et al*., 2018). Furthermore, studies have reported that one-third of patients with CA-CDI have no apparent exposure to traditional risk factors of hospitalization or antimicrobials (Mooney *et al*., 2008). The driving factors behind the shift towards CA-CDI are not well understood, making infection prevention and control, and establishing effective eradication programs challenging.

To date, *C. difficile* has been detected in a diverse range of sources from compost and lawns to root vegetables and livestock (Moono *et al*., 2017; Lim *et al*., 2018a; Lim *et al*., 2018b). The presence of *C. difficile* in production animals has been investigated, with evidence of overlap of strains seen in animals and humans (Songer *et al*., 2009; Medina-Torres *et al*., 2011; Knight and Riley, 2013). The emergence of human disease caused by strains previously only seen in animals also adds to mounting evidence that CDI may be zoonotic, highlighting a need for a holistic One Health approach to understand and control this disease (Knight *et al*., 2015b; Rodriguez *et al*., 2016).

The 'One Health' paradigm recognizes the relationship between human health, animal health and the environment. It highlights the need to review all factors contributing to a health issue in attempts to control, prevent and treat disease. The ubiquitous nature of *C. difficile* makes a One Health approach vital to public health planning. Interaction between the main constituents influencing *C. difficile* transmission (seen in Fig. 2) is extensive and complicated. Horses represent an interesting addition in the *C. difficile* story, with overlapping domains of production animal, companion animal and non-domesticated populations. The potential for dissemination of *C. difficile* encompasses transfer through interaction, consumption and indirectly through exposure to horse manure. In countries such as Australia, with an estimated 400 000 feral horses, there is also significant potential for dissemination at the wildlife–livestock–human interface through interaction with other wild and native species, as well as dispersal through shared water sources and possible

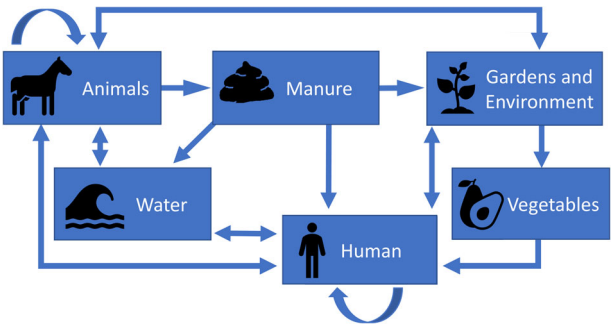


**Fig. 2.** The proposed interaction of elements in *C. difficile* dispersion.

interaction with free-range livestock (Csurhes *et al*., 2016). To date, *C. difficile* in feral horses and the role they play in dispersal has not been investigated. It was previously noted that commercially available animal manures and compost showed traces of *C. difficile*. Local horse manure is readily available for use in domestic and market gardens, and on farms is often furrowed back into paddocks allowing the potential for the spread of any existing *C. difficile* spores into the community. This creates a potential pathway for transfer between horses and humans.

Furthermore, the emergence of *C. difficile* AMR in both human and animal strains and the possibility of bidirectional resistance gene transfer between the two add a further complication to the system (Knetsch *et al*., 2018). Studies have shown horse derived *C. difficile* strains show high resistance to commonly used antimicrobials such as ceftiofur and gentamicin, suggesting AMR may be influenced by their use (Rodriguez *et al*., 2014). Injudicious use of antimicrobials in human healthcare, veterinary practices and farming has come under great scrutiny for its contribution to AMR and the spread of infectious disease. In 2015, the World Health Assembly identified AMR as a critical issue, endorsing the development of a global action plan to tackle this problem. In addition, in 2013 and 2019, the US Centers for Disease Control and Prevention identified *C. difficile* in the top five microorganisms posing an urgent threat to public health due to its developing AMR (Centers for Disease Control and Prevention, 2019). Given the current global crisis of AMR and the pervasiveness of *C. difficile*, it is vital that the health and science communities start to look outside their immediate fields for solutions. For this reason, investigations into the aetiology and epidemiology of *C. difficile* in non-traditional sources are required.

## The way forward

Despite promising developments in the understanding of equine *C. difficile*, a lack of validation for species-specific diagnostic testing and treatment, as well as the ongoing threat of AMR, creates challenges in the fight against CDI. The implications of asymptomatic carriage on the dispersal of the bacterium also remains elusive and strain characterization and molecular investigation may prove crucial for a true appreciation of the *C. difficile* epidemiology to assist in tracing the flow through horse populations and the community as a whole. As the links between *C. difficile* in animals, humans and the environment become increasingly apparent, a more efficient approach to antimicrobial surveillance, stewardship and AMR investigations is needed for long-term sustainability. A One Health approach and further appreciation of the possible sources of *C. difficile* are therefore vital to the development of infection prevention and control strategies, to minimize transmission risk as well as generate protocols for optimal antimicrobial use.

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