Clostridium difficile in horses in Australia – a preliminary study

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During a 24 month period from 2007 to 2009, 174 faecal specimens from horses in Australia (predominantly from Western Australia) were tested for *Clostridium difficile*. *C. difficile* was isolated from 14 (23 %) of 62 diarrhoeal animals (including 10 foals) and from none of 112 healthy adult horses. These isolates were toxin profiled by PCR for toxin A, toxin B and binary toxin, and ribotyped. Ten of the equine isolates were $A^+B^+CDT^-$. Other toxin profiles detected were $A^-B^-CDT^+$ (one isolate), $A^+B^+CDT^+$ (two isolates) and $A^-B^-CDT^-$ (three isolates). There were six different ribotypes detected in the horses, ribotype 012 being the most common with six isolates. Two horses (one adult and one foal) had two strains of *C. difficile* isolated on different days. These strains had the same toxin profile but different ribotypes. None of the equine isolates was ribotype 078, which is $A^+B^+CDT^+$ and a significant cause of animal disease overseas. All isolates were susceptible to metronidazole and vancomycin. These results suggest that the epidemiology of *C. difficile* in horses in Australia is currently similar to that in other parts of the world, but requires further surveillance to monitor changes.

INTRODUCTION

The anaerobic bacterium *Clostridium difficile* is a major human nosocomial pathogen, the most commonly diagnosed cause of infectious hospital diarrhoea (Riley, 1998). In humans *C. difficile* infection (CDI) has a wide clinical spectrum, ranging from asymptomatic carriage, to mild-self-limiting diarrhoea, and more severe pseudomembranous colitis. Toxigenic isolates of *C. difficile* usually produce two toxins, toxin A and toxin B, and these are thought of as the major virulence factors. Some strains of *C. difficile* produce an additional toxin, binary toxin (actin-specific ADP-ribosyl-transferase, CDT), first reported in 1988 but not considered important until now (Riley, 2006). In both North America and Europe, a hypervirulent strain of *C. difficile* (PCR ribotype 027) has appeared in the last few years resulting in high mortality and morbidity in humans. This strain is resistant to fluoroquinolone antibiotics, and use of fluoroquinolones appears to be driving the epidemic (Riley, 2006). In an epidemic in Canada, on average, each case resulted in an additional 10.7 days in hospital and, compared with controls, patients with CDI had a significantly higher mortality of >10 % in those aged 70 years or more (Loo *et al.*, 2005).

There has been speculation that *C. difficile* may be zoonotic and that transmission of infection via spores may be food borne (Rupnik, 2007). *C. difficile* is known to colonize many animals (Levett, 1986). Indeed, as with humans, the gastrointestinal tracts of most infant animals are probably colonized by *C. difficile* until weaning. In the last 20 years, *C. difficile* has become accepted as an enteric pathogen in horses, particularly in Europe (Båverud, 2004) and North America (Weese *et al.*, 2001) but not universally in Australia. The bacterium is associated with acute colitis in mature horses usually following treatment with antibiotics (Båverud *et al.*, 1997; Ruby *et al.*, 2009). *C. difficile*, and/or its cytotoxin, is also associated with acute colitis in mares when their foals are being treated with erythromycin and rifampicin for *Rhodococcus equi* pneumonia (Båverud *et al.*, 1998). *C. difficile* can be isolated from foals with diarrhoea (Jones *et al.*, 1987), although foals under the age of 2 weeks are often colonized with *C. difficile* with no apparent disease (Båverud *et al.*, 2003).

There are no data on *C. difficile* disease in horses in Australia. This paper presents a preliminary investigation into the prevalence of *C. difficile* in horses with and without diarrhoea in Australia. In addition to determining prevalence, a further objective was to determine the production of toxins by and the antibiotic susceptibility patterns of any strains isolated.

Abbreviations: CDI, *Clostridium difficile* infection; EI, equine influenza.

METHODS

Samples. Faecal samples were collected from horses with (n=62) and without (n=112) diarrhoea. Samples from diarrhoeal horses and

foals were received from veterinary clinics in Western Australia and New South Wales. These samples were from cases of sporadic diarrhoea with no evidence of any association with an outbreak. Samples from normal healthy adult horses without diarrhoea were collected from stud and agistment (livery) farms predominantly in Western Australia but also from other Australian states. The project was severely impacted by the equine influenza (EI) outbreak in Australia in the second half of 2007. The EI outbreak meant that samples from other Australian states could not be transported to Western Australia. Permission was granted from the Western Australian Quarantine Inspection Service to start importing samples into Western Australia again in late 2008.

Environmental samples were collected from the Murdoch University Veterinary Hospital (Murdoch, Western Australia) section that treats horses, as other work has shown that veterinary clinics can become grossly contaminated with *C. difficile*, potentially posing a risk to animals and veterinarians (Båverud, 2004).

Detection of *C. difficile*. Faecal samples were plated onto selective agar plates [cycloserine cefoxitin fructose agar (CCFA)] and incubated anaerobically for 48 h in a Don Whitley Scientific anaerobic chamber (Bowman & Riley, 1988). In addition to solid media, an enrichment broth containing gentamicin, cycloserine and cefoxitin (GCC broth) was employed as described previously (Carroll *et al.*, 1983). Environmental samples were cultured in and on media containing sodium taurocholate, as this has been shown to enhance germination of *C. difficile* spores. Putative colonies of *C. difficile* were confirmed by their characteristic appearance and odour, followed by a specific latex particle agglutination (Bowman *et al.*, 1986). Isolates were confirmed as *C. difficile* by a species-specific PCR that also identified the presence of toxin A (*tcdA*), toxin B (*tcdB*) and binary toxin genes (*cdtA* and *cdtB*). Antimicrobial susceptibility patterns were determined by agar dilution and Etest strip (bioMérieux).

Typing of isolates. For accurately determining the epidemiology of infection it is necessary to type isolates from animals and the environment to show how they are related. Although a variety of different typing methods have been developed for *C. difficile* (Brazier, 2001), PCR ribotyping (Stubbs *et al.*, 1999) has gained widespread acceptance as the method of choice in Europe and continues to be applied throughout the world. This technique was applied to all isolates to look for relatedness and clusters.

Risk factors. When available, data were collected on antibiotic exposure and other potential risk factors for *C. difficile*.

RESULTS AND DISCUSSION

The overall prevalence of *C. difficile* in the diarrhoeal horses studied was 23 % (14/62). *C. difficile* was found only in this group, and in none of 112 normal healthy adult horses (82 from Western Australia and 30 from New South Wales). In relation to the horses (adults and foals) with diarrhoea, of the 5 (11 %) positive samples from 47 samples from Western Australia, 3 were from adults and 2 were from foals aged 5 and 6 months, while of the 9 (60 %) positive samples from 15 samples from New South Wales, 1 was an adult and 8 were foals. All New South Wales foals were less than 2 weeks old and seven of the nine positive horses in New South Wales had been given antibiotics. A total of 43 environmental swabs was taken from surfaces in the Murdoch University veterinary clinic equine section (treatment areas/isolation areas) and none was positive for *C. difficile*.

Fig. 1 shows a dendrogram containing all the strains of *C. difficile* isolated together with their toxin profiles. Ten of the equine isolates were $A^+B^+CDT^-$. Other toxin profiles detected were $A^-B^-CDT^+$ (one isolate), $A^+B^+CDT^+$ (two) and $A^-B^-CDT^-$ (three). There were six different ribotypes detected, ribotype 012 being the most common with six isolates. Two horses (one adult and one foal) had two different strains of *C. difficile* isolated on different days. These strains had the same toxin profile but were different ribotypes. The most common PCR ribotype recovered was PCR ribotype 012, which comprised 6 of the 16 isolates (37.5 %). The only other ribotype identified with more than 1 isolate was PCR ribotype 014 with 2 of 16 (12.5 %).

Antibiotic susceptibility data for the 16 isolates of *C. difficile* are given in Table 1. There was no vancomycin or metronidazole resistance detected, with MICs generally extremely low for both antimicrobials. In addition, there was no high level (MICs >128 mg l^{-1}) resistance to clindamycin. Three strains showed high-level resistance to levofloxacin, two of which were also resistant to moxifloxacin. One strain showed resistance to penicillin, and this requires further investigation, while several strains showed borderline resistance to this drug.

There are a number of causes of diarrhoea in the horse, both infectious and non-infectious. The infectious agents most commonly isolated from horses with colitis are *C. difficile*, *Clostridium perfringens* and *Salmonella* spp. (Weese *et al.*, 2001; Weese, 2002). Horses also get colitis following antibiotic therapy, 'antibiotic-associated diarrhoea' and, as with humans, this is often associated with *C. difficile* (Madewell *et al.*, 1995; Weese *et al.*, 2001; Ruby *et al.*, 2009). Despite this mounting evidence, there has been some scepticism in Australian equine circles about any role that *C. difficile* might play in colitis in horses. This is perhaps not surprising as a role for *C. difficile* in diarrhoeal disease in horses is only a relatively recent finding. Indeed, the epidemiology of CDI in production and companion animals has changed rapidly over the last 10–20 years. Whereas *C. difficile* was never thought of as a pathogen or colonizer of pigs, cows or turkeys in the past, a recently published survey of retail pork, beef and turkey in the USA indicated that 40 % of samples were contaminated with *C. difficile* (Songer *et al.*, 2009b). In addition, *C. difficile* causes clinical disease in neonatal piglets and is now the leading cause of neonatal scours in these animals, resulting in considerable morbidity and mortality, and subsequent economic losses for the industry (Songer *et al.*, 2000). In companion animals the situation was a little different with 40 % of animals attending a veterinary clinic in Perth, Western Australia, in the early 1990s colonized with *C. difficile*. However, these strains did not appear to be infecting humans (Riley *et al.*, 1991). Today the situation is different, and human infection, apparently with animal strains of *C. difficile*, is common in Europe (Goorhuis *et al.*, 2008).

This project represents the first survey of its kind in Australia. Even though the number of samples was limited,

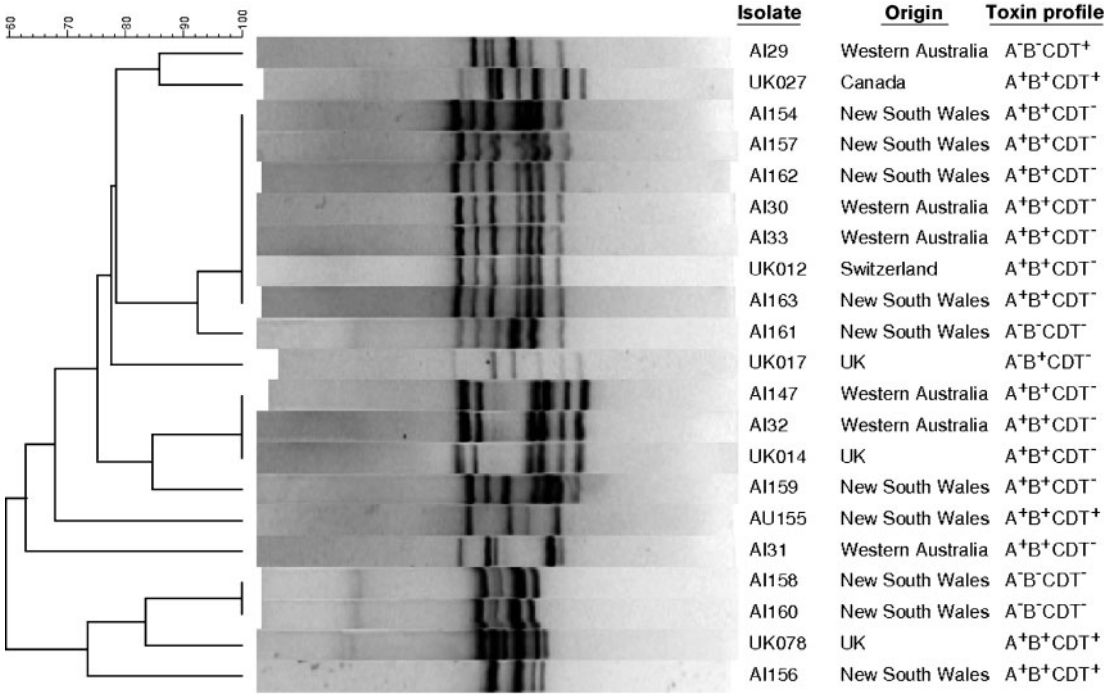

Fig. 1. PCR ribotypes of strains of *C. difficile* isolated in Australia from horses. Representative strains of various control PCR ribotypes are shown for comparison. The toxin profile for each isolate is also indicated.

and the EI outbreak in 2007–2008 impacted on the availability of samples, the results are nonetheless very revealing. There is now no doubt that *C. difficile* can be found in Australian horses and is apparently associated with diarrhoeal disease, although further better controlled studies will be required to document its exact role. In the

Table 1. Antibiotic susceptibility data for isolates of *C. difficile* from Australian horses

Isolate no.	MICs for the antibiotics shown						
	Moxifloxacin ($\geqslant$4 mg l^{-1}*)	Clindamycin ($\geqslant$4 mg l^{-1}*)	Levofloxacin ($\geqslant$2 mg l^{-1}*)	Metronidazole ($\geqslant$8 mg l^{-1}*)	Vancomycin ($\geqslant$4 mg l^{-1}*)	Penicillin ($\geqslant$2 mg l^{-1}*)	Ciprofloxacin ($\geqslant$2 mg l^{-1}*)
AI29	0.75	4	2	0.25	0.5	1	6
AI30	>32	8	>32	0.094	0.38	2	>32
AI31	0.5	6	2	0.094	0.75	1	4
AI32	2	2	>32	0.094	0.38	>32	>32
AI33	>32	4	>32	0.064	0.38	1.5	>32
AI147	0.5	6	3	0.19	0.25	1.5	8
AI154	0.5	12	4	0.125	0.75	2	>32
AI155	0.75	4	4	0.38	0.75	1	6
AI156	0.5	0.032	3	0.19	0.5	1	4
AI157	0.38	12	4	0.047	0.5	2	>32
AI158	0.5	6	3	0.38	0.5	0.75	8
AI159	0.38	6	6	0.064	0.75	2	8
AI160	0.38	3	3	0.25	0.5	2	6
AI161	0.38	1	3	0.094	0.75	2	3
AI162	0.5	8	4	0.047	0.38	2	6
AI163	0.25	6	4	0.047	0.38	2	6

*Susceptibility breakpoints.

present study, it was not possible to culture all diarrhoeal samples for other enteric pathogens, and information on whether this had been done elsewhere was not always available.

However, the overall isolation rate for *C. difficile* in diarrhoeal horses was 23 %, which compares favourably with the publication by Båverud (2004) in which the prevalence was 42 % in horses with acute colitis during antibiotic treatment. *C. difficile* may also have a role in other gastrointestinal diseases in horses. Arroyo *et al.* (2006) recently reported *C. difficile* as a possible cause of duodenitis–proximal jejunitis in horses, and colitis X in horses has been associated with the isolation of *C. difficile* (Songer *et al.*, 2009a). The role of *C. difficile* in disease in foals in the absence of antimicrobial exposure is less clear. There are several reports of CDI in foals but the picture is different to adult horses with diarrhoea in the absence of antibiotic therapy (Magdesian *et al.*, 2002). This is not unique to horses. *C. difficile* can also be isolated from calves with and without diarrhoea with equal frequency (Rodriguez-Palacios *et al.*, 2006). It is likely that all young animals are colonized with *C. difficile* from birth. Once a more adult gastrointestinal microflora is established then *C. difficile* cannot compete and is eliminated. However, it is still possible that *C. difficile* causes disease in young animals, with piglets being the prime example.

None of 43 environmental swabs taken from surfaces in the Murdoch University equine section (treatment areas/isolation areas) was positive for *C. difficile*. This was a little surprising given the widespread environmental contamination that occurs with *C. difficile* in the veterinary setting (Weese *et al.*, 2000; Båverud *et al.*, 2003); however, what number of horses had been through the facility and how much cleaning had occurred afterwards was not known. Certainly the facility looked very clean to the naked eye.

The ribotypes of *C. difficile* recovered from horses in this study were very similar to those isolated from humans in Australia (B. Elliott and T. V. Riley, unpublished data). Ribotype 014 is the most common ribotype (17 %) of *C. difficile* isolated from humans in Australia, while ribotype 012 is also quite common. The sharing of ribotypes between horses and humans has been reported before and indeed appears quite common (Keel *et al.*, 2007). This is different to the situation with cattle/pigs and humans where, until recently, most cattle/pig isolates of *C. difficile* were ribotype 078, a type rarely found in humans previously. This has now changed with ribotype 078, apparently from pigs, now a common ribotype of *C. difficile* isolated from humans in Europe (Goorhuis *et al.*, 2008; Bauer *et al.*, 2011). Clearly the possibility of the transfer of animal strains of *C. difficile* to humans exists, as does the reverse, and this should be a cause for concern by anyone involved in production/companion animal industries.

Most strains of *C. difficile* isolated in this study remained susceptible to the majority of antibiotics. One cause for concern was the two isolates (12.5 %) that were resistant to moxifloxacin. Fluoroquinolone resistance in certain strains of *C. difficile* and overuse of fluoroquinolones is driving the current human *C. difficile* epidemic overseas. *C. difficile* quickly mutates to a state of fluoroquinolone resistance after exposure to this group of antibiotics through changes to the *gyrA* and *gyrB* genes (Ackermann *et al.*, 2003). No metronidazole resistance was seen, in contrast to some reports in both humans (Peláez *et al.*, 2002) and horses (Magdesian *et al.*, 2002).

It may also be worth mentioning that there has been little evaluation of the methods for detecting *C. difficile* in horse faecal samples. A number of enzyme immunoassay kits are marketed for this purpose in humans; however, these have not been evaluated extensively in horses (Medina-Torres *et al.*, 2010). In the present study, samples from 10 of the 14 horses from which *C. difficile* was isolated were evaluated with the C. diff Quik Chek Complete kit (TechLab). Nine of the ten were positive for the GDH component of the kit while only one gave a positive for the toxinA/B component of the kit (S. Thean & T. V. Riley, data not shown). Many human diagnostic laboratories are moving to molecular methods with either in-house or commercially available tests to detect mainly the toxin B gene in *C. difficile*. Again there has been little molecular diagnostic work with horses and this may be a fruitful avenue for research in the future.

In summary, *C. difficile* is present in Australian adult horses and foals with diarrhoea. What role the organism plays, particularly in diarrhoea in foals, requires further investigation. However, the evidence from this study and the literature suggests *C. difficile* may be an important cause of diarrhoea in adult horses and the industry should not ignore this.

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REFERENCES

Ackermann, G., Tang-Feldman, Y. J., Schaumann, R., Henderson, J. P., Rodloff, A. C., Silva, J. & Cohen, S. H. (2003). Antecedent use of fluoroquinolones is associated with resistance to moxifloxacin in *Clostridium difficile*. *Clin Microbiol Infect* **9**, 526–530.

Arroyo, L. G., Stämpfli, H. R. & Weese, J. S. (2006). Potential role of *Clostridium difficile* as a cause of duodenitis-proximal jejunitis in horses. *J Med Microbiol* **55**, 605–608.

Bauer, M. P., Notermans, D. W., Van Benthem, B. H. B., Brazier, J. S., Wilcox, M. H., Rupnik, M., Monnet, D. L., Van Dissel, J. T. & Kuijper, E. J. for the ECDIS Study Group (2011). *Clostridium difficile* infection in Europe: a hospital-based survey. *Lancet* **377**, 63–73.

Båverud, V. (2004). *Clostridium difficile* diarrhea: infection control in horses. *Vet Clin North Am Equine Pract* **20**, 615–630.

Båverud, V., Gustafsson, A., Franklin, A., Lindholm, A. & Gunnarsson, A. (1997). *Clostridium difficile* associated with acute colitis in mature horses treated with antibiotics. *Equine Vet J* **29**, 279–284.

Båverud, V., Franklin, A., Gunnarsson, A., Gustafsson, A. & Hellander-Edman, A. (1998). *Clostridium difficile* associated with acute colitis in mares when their foals are treated with erythromycin and rifampicin for *Rhodococcus equi* pneumonia. *Equine Vet J* **30**, 482–488.

Båverud, V., Gustafsson, A., Franklin, A., Aspán, A. & Gunnarsson, A. (2003). *Clostridium difficile:* prevalence in horses and environment, and antimicrobial susceptibility. *Equine Vet J* **35**, 465–471.

Bowman, R. A. & Riley, T. V. (1988). Laboratory diagnosis of *Clostridium difficile*-associated diarrhoea. *Eur J Clin Microbiol Infect Dis* **7**, 476–484.

Bowman, R. A., Arrow, S. A. & Riley, T. V. (1986). Latex particle agglutination for detecting and identifying *Clostridium difficile. J Clin Pathol* **39**, 212–214.

Brazier, J. S. (2001). Typing *of Clostridium difficile. Clin Microbiol Infect* **7**, 428–431.

Carroll, S. M., Bowman, R. A. & Riley, T. V. (1983). A selective broth for *Clostridium difficile. Pathology* **15**, 165–167.

Goorhuis, A., Bakker, D., Corver, J., Debast, S. B., Harmanus, C., Notermans, D. W., Bergwerff, A. A., Dekker, F. W. & Kuijper, E. J. (2008). Emergence of *Clostridium difficile* infection due to a new hypervirulent strain, polymerase chain reaction ribotype 078. *Clin Infect Dis* **47**, 1162–1170.

Jones, R. L., Adney, W. S. & Shideler, R. K. (1987). Isolation of *Clostridium difficile* and detection of cytotoxin in the feces of diarrheic foals in the absence of antimicrobial treatment. *J Clin Microbiol* **25**, 1225–1227.

Keel, K., Brazier, J. S., Post, K. W., Weese, S. & Songer, J. G. (2007). Prevalence of PCR ribotypes among *Clostridium difficile* isolates from pigs, calves, and other species. *J Clin Microbiol* **45**, 1963–1964.

Levett, P. N. (1986). *Clostridium difficile* in habitats other than the human gastro-intestinal tract. *J Infect* **12**, 253–263.

Loo, V. G., Poirier, L., Miller, M. A., Oughton, M., Libman, M. D., Michaud, S., Bourgault, A. M., Nguyen, T., Frenette, C. & other authors (2005). A predominantly clonal multi-institutional outbreak of *Clostridium difficile*-associated diarrhea with high morbidity and mortality. *N Engl J Med* **353**, 2442–2449.

Madewell, B. R., Tang, Y. J., Jang, S., Madigan, J. E., Hirsh, D. C., Gumerlock, P. H. & Silva, J., Jr (1995). Apparent outbreaks of *Clostridium difficile*-associated diarrhea in horses in a veterinary medical teaching hospital. *J Vet Diagn Invest* **7**, 343–346.

Magdesian, K. G., Hirsh, D. C., Jang, S. S., Hansen, L. M. & Madigan, J. E. (2002). Characterization of *Clostridium difficile* isolates from foals with diarrhea: 28 cases (1993–1997). *J Am Vet Med Assoc* **220**, 67–73.

Medina-Torres, C. E., Weese, J. S. & Staempfli, H. R. (2010). Validation of a commercial enzyme immunoassay for detection of *Clostridium difficile* toxins in feces of horses with acute diarrhea. *J Vet Intern Med* **24**, 628–632.

Peláez, T., Alcalá, L., Alonso, R., Rodríguez-Créixems, M., García-Lechuz, J. M. & Bouza, E. (2002). Reassessment of *Clostridium difficile* susceptibility to metronidazole and vancomycin. *Antimicrob Agents Chemother* **46**, 1647–1650.

Riley, T. V. (1998). *Clostridium difficile*: a pathogen of the nineties. *Eur J Clin Microbiol Infect Dis* **17**, 137–141.

Riley, T. V. (2006). Epidemic *Clostridium difficile. Med J Aust* **185**, 133–134.

Riley, T. V., Adams, J. E., O'Neill, G. L. & Bowman, R. A. (1991). Gastrointestinal carriage of *Clostridium difficile* in cats and dogs attending veterinary clinics. *Epidemiol Infect* **107**, 659–665.

Rodriguez-Palacios, A., Stämpfli, H. R., Duffield, T., Peregrine, A. S., Trotz-Williams, L. A., Arroyo, L. G., Brazier, J. S. & Weese, J. S. (2006). *Clostridium difficile* PCR ribotypes in calves, Canada. *Emerg Infect Dis* **12**, 1730–1736.

Ruby, R., Magdesian, K. G. & Kass, P. H. (2009). Comparison of clinical, microbiologic, and clinicopathologic findings in horses positive and negative for *Clostridium difficile* infection. *J Am Vet Med Assoc* **234**, 777–784.

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

Songer, J. G., Post, K. W., Larson, D. J., Jost, B. H. & Glock, R. D. (2000). Infection of neonatal swine with *Clostridium difficile. Swine Health Prod* **8**, 185–189.

Songer, J. G., Trinh, H. T., Dial, S. M., Brazier, J. S. & Glock, R. D. (2009a). Equine colitis X associated with infection by *Clostridium difficile* NAP1/027. *J Vet Diagn Invest* **21**, 377–380.

Songer, J. G., Trinh, H. T., Killgore, G. E., Thompson, A. D., McDonald, L. C. & Limbago, B. M. (2009b). *Clostridium difficile* in retail meat products, USA, 2007. *Emerg Infect Dis* **15**, 819–821.

Stubbs, S. L., Brazier, J. S., O'Neill, G. L. & Duerden, B. I. (1999). PCR targeted to the 16S-23S rRNA gene intergenic spacer region of *Clostridium difficile* and construction of a library consisting of 116 different PCR ribotypes. *J Clin Microbiol* **37**, 461–463.

Weese, J. S. (2002). A review of equine zoonotic diseases: risks in veterinary medicine. *AAEP Proceedings* **48**, 362–369.

Weese, J. S., Staempfli, H. R. & Prescott, J. F. (2000). Isolation of environmental *Clostridium difficile* from a veterinary teaching hospital. *J Vet Diagn Invest* **12**, 449–452.

Weese, J. S., Staempfli, H. R. & Prescott, J. F. (2001). A prospective study of the roles of *Clostridium difficile* and enterotoxigenic *Clostridium perfringens* in equine diarrhoea. *Equine Vet J* **33**, 403–409.