

Clostridium difficile: prevalence in horses and environment, and antimicrobial susceptibility

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Summary

Reasons for performing study: *Clostridium difficile* has been associated with acute colitis in mature horses.

Objectives: To survey *C. difficile* colonisation of the alimentary tract with age, occurrence of diarrhoea and history of antibiotic therapy; and to study the occurrence and survival of *C. difficile* in the environment and antimicrobial susceptibility of isolated strains.

Methods: A total of 777 horses of different breeds, age and sex were studied. Further, 598 soil samples and 434 indoor surface samples were examined. Antimicrobial susceptibility of 52 strains was investigated by Etest for 10 antibiotics.

Results: In horses that developed acute colitis during antibiotic treatment, 18 of 43 (42%) were positive to *C. difficile* culture and 12 of these (28%) were positive in the cytotoxin B test. Furthermore, *C. difficile* was isolated from a small number of diarrhoeic mature horses (4 of 72 [6%]) with no history of antibiotic treatment, but not from 273 healthy mature horses examined or 65 horses with colic. An interesting new finding was that, in normal healthy foals age <14 days, *C. difficile* was isolated from 1/3 of foals (16 of 56 [29%]). All older foals (170) except one were negative. Seven of 16 (44%) nondiarrhoeic foals treated with erythromycin or gentamicin in combination with rifampicin were also excretors of *C. difficile*.

On studfarms, 14 of 132 (11%) outdoor soil samples were positive for *C. difficile* in culture, whereas only 2 of 220 (1%) soil samples from farms with mature horses were positive for *C. difficile* ($P = <0.001$). By PCR, it was demonstrated that strains from the environment and healthy foals can serve as a potential reservoir of toxigenic *C. difficile*. The experimental study conducted here found that *C. difficile* survived in nature and indoors for at least 4 years in inoculated equine faeces. The susceptibility of 52 strains was investigated for 10 antibiotics and all were susceptible to metronidazole (MIC \leq 4 mg/l) and vancomycin (MIC \leq 2 mg/l).

Conclusions: *C. difficile* is associated with acute colitis in mature horses, following antibiotic treatment. Furthermore, *C. difficile* was isolated from 1 in 3 normal healthy foals age <14 days.

Potential relevance: Strains from healthy foals and the environment can serve as a potential reservoir of toxigenic *C. difficile*.

Introduction

Acute colitis in mature horses is a disease associated with high mortality, but its aetiology often remains unclear. Different bacterial pathogens have been proposed to be the causative agent (Mair *et al.* 1990; Staempfli *et al.* 1991; Murray 1992; Palmer 1992). *Clostridium difficile* was suggested recently as a possible enteric pathogen in mature horses (Jang *et al.* 1997; Donaldson and Palmer 1999; Weese *et al.* 2001) in association with antibiotic treatment (Beier *et al.* 1994; Cosmetatos *et al.* 1994; Madewell *et al.* 1995; Båverud *et al.* 1997, 1998; Gustafsson *et al.* 1997). In human medicine, *C. difficile* is a well-known cause of nosocomial diarrhoea induced by antibiotic treatment (Tabaqchali and Jumaa 1995; Job and Jacobs 1997).

In man, *C. difficile* frequently colonises the intestine of infants age less than 18 months (Merida *et al.* 1986; Tullus *et al.* 1989). This condition was regarded as harmless until reports recently appeared of *C. difficile* causing disease in infants (Kelly *et al.* 1994; McGowan and Kader 1999). In young foals, age 1–20 days, some authors have reported an association between *C. difficile* and diarrhoea (Jones *et al.* 1987, 1988a,b; Magdesian *et al.* 1999). In Sweden, *C. difficile* has not been found in faecal samples of healthy untreated foals in the age range 14 days–4 months (Båverud *et al.* 1998) whereas, in the USA, it has been reported in a low frequency (Jones *et al.* 1987; Magdesian *et al.* 1999). Several studies have shown that the organism is widespread on human hospital wards and on hands of hospital staff (McFarland *et al.* 1989; Al Saif and Brazier 1996). Few studies have been directed at the environment outside hospitals.

In horses, colitis associated with *C. difficile* is a new issue. We need to learn more about the pathogenesis, epidemiology and survival of this bacterium in the environment and its susceptibility to antibiotics. The aims of the present investigation were to study, in horses, the association of *C. difficile* colonisation with age, occurrence of diarrhoea and antibiotic treatment. The survival and occurrence of the bacterium in the environment and its antimicrobial susceptibility were also studied.

Materials and methods

A total of 777 horses of different breeds, age and sex were included in the study, divided into categories of mature horses and foals. The

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TABLE 1: Prevalence of *Clostridium difficile* and demonstration of cytotoxin B in different clinical categories of mature horses

Group	No. horses	No. (%) of horses positive for <i>C. difficile</i> by:		
		Culture	Cytotoxin B test*	Culture ± cytotoxin B test
A: Horses with no signs of enteric disorders	273	0 (0)	0 (0)	0 (0)
B: Horses with acute colitis during antibiotic treatment	43	18 (42)	12 (28)	18 (42)
C: Horses with colitis and no antibiotic treatment	72	4 (6)	0 (0)	4 (6)
D: Horses with no signs of enteric disorders and treated with antibiotics	47	1 (2)	0 (0)	1 (2)
E: Horses with colic, no diarrhoea and no antibiotic treatment	65	0 (0)	0 (0)	0 (0)
Total	500	23	12	23

*Group A 247 horses; Group B all horses; Group C 65 horses; Group D 38 horses and Group E 34 horses tested.

500 mature horses were subdivided into 5 groups (A–E; Table 1) and the 277 foals into 4 groups (1–4; Tables 2, 3).

Mature horses

Group A - no signs of enteric disorders: This group (n = 273) comprised 40 horses from 3 riding schools, 25 private riding horses, 51 trotters from 3 camps, 60 broodmares from 4 stables, 37 horses belonging to the Faculty of Veterinary Medicine (FVM), Uppsala, Sweden, used in teaching, and 60 horses hospitalised at 3 animal hospitals.

Group B - acute colitis during antibiotic treatment: These horses (n = 43) had developed watery, foul-smelling diarrhoea with acute onset following treatment with antibiotics for other disorders. Other clinical signs were fever, discoloured mucous membranes, tachycardia, depression and anorexia. The diarrhoea frequently resulted in metabolic acidosis, profound dehydration, signs of toxæmia and hypovolaemia. Of these 43 horses from 3 animal hospitals, 29 developed diarrhoea when hospitalised. Four horses developed diarrhoea at their home stable after having recently visited an animal hospital and 10 horses developed diarrhoea at their home stable without having visited a clinic or animal hospital. Before the onset of acute colitis, antibiotics administered included procaine penicillin, benzylpenicillin, ampicillin, a combination of dihydrostreptomycin sulphate and procaine penicillin, gentamicin, tetracycline, trimethoprim/sulphadiazine (TMP/SDZ) and bacitracin, alone or in combination.

Group C - colitis, no previous antibiotic treatment: Of this group (n = 72), 66 horses developed diarrhoea primarily at their home stable and another 6 horses at an animal hospital. A majority of the cases (43) were classified as acute and the others as subacute to chronic. Samples from 55 horses were taken at 3 animal hospitals and 3 large animal clinics and 17 horses were sampled in general practice.

Group D - no signs of enteric disorders, treated with antibiotics: Samples were taken at 3 animal hospitals after at least 3 days of antibiotic treatment for disorders other than diarrhoea. The horses in this group (n = 47) were treated with procaine penicillin,

TABLE 2: Prevalence of *Clostridium difficile* in healthy untreated foals (Group 1) of different ages

Age groups	No. horses	No. (%) horses positive for <i>C. difficile</i> by culture	No. positive isolates for toxin A and B, by PCR/No. tested isolates
0–13 days	56	16 (29)	5/14
14–29 days	37	1 (3)	1/1
30–59 days	41	0 (0)	-
60–89 days	38	0 (0)	-
3–6 months	54	0 (0)	-
Total	226	17 (8)	6/15

benzylpenicillin, ampicillin, gentamicin, TMP/SDZ, a combination of dihydrostreptomycin and procaine penicillin and metronidazole, alone or in combination.

Group E - colic but no diarrhoea, no previous antibiotic treatment: The horses (n = 65) were admitted to the FVM with a primary sign of colic. Samples were normally taken during the first or second day of hospitalisation. The horses in this group did not develop diarrhoea and were not treated with antibiotics.

Foals

A total of 277 nursing foals of different breeds were included in the present study, age 0 days–7 months. They were subdivided into 4 major groups based on clinical status.

Group 1 - foals apparently healthy, not treated with antibiotics: All foals (n = 226), age 0 day–6 months, were subdivided into 5 age categories (Table 2). They were from 8 studfarms from different parts of the country.

Group 2 - foals with no diarrhoea, treated with erythromycin or gentamicin in combination with rifampicin: Of this group (n = 16), 11 foals, age 1–3 months, were treated with erythromycin and 5 with gentamicin, both in combination with rifampicin, for *Rhodococcus equi* infection (Båverud *et al.* 1998). The foals stayed at animal hospitals (Table 3).

Group 3 - foals with no diarrhoea treated with penicillin and/or TMP/SDZ: Within this group (n = 13), 8 foals were age 0–13 days, 2 age 14–29 days and 3 age 1 month. The foals were treated with penicillin (procaine and benzylpenicillin) and/or TMP/SDZ for diseases other than gastrointestinal. All foals were sampled in general practice (Table 3).

Group 4 - foals with diarrhoea: This was a heterogeneous group (n = 22) with diarrhoea as a common sign. A majority of the cases (n = 14) were classified as acute and the others as subacute to chronic. The following antibiotics were used; erythromycin in combination with rifampicin, procaine penicillin, benzylpenicillin, TMP/SDZ, tetracycline, a combination of dihydrostreptomycin and procaine penicillin and gentamicin, alone or in combination. Twelve foals were sampled at animal hospitals and 10 in general practice (Table 3).

Bacteriological and toxicological investigations of faeces

Sampling of faeces: Faecal samples were taken from the rectum and packed in plastic tubes or thick plastic bags. Excess air was

TABLE 3: Prevalence of *Clostridium difficile* in diseased foals of different categories

Group	No. foals	No. (%) horses positive for <i>C. difficile</i> by culture
2: Foals with no diarrhoea, treated with erythromycin or gentamicin + rifampicin, age 1–3 months	16	7 (44)
3: Foals with no diarrhoea, treated with penicillin and/or TMP/SDZ, age 0–13 days	8	2 (25)
age 14 days–1 month	5	0 (0)
4: Foals with diarrhoea, age 5 days–7 months*	22	2† (9)
Total	51	11

*A heterogeneous group. Nine foals treated, 8 not treated with antibiotics and for 5 there was no report. †The 2 *C. difficile*-positive foals were ages 20 days and 5 months. The first foal was treated with benzylpenicillin, trimethoprim/sulphadiazine (TMP/SDZ) and erythromycin in combination with rifampicin; the second foal with procaine penicillin, benzylpenicillin and TMP/SDZ.

evacuated. All samples were cultured and frozen (-20°C) within 48 h (most within 8 h) and tested subsequently for cytotoxin B. Most foals were sampled with rectal swabs which were transported to the laboratory in Amies medium with charcoal¹ and cultured within 24 h. The faecal swabs from foals were not tested for cytotoxin B because the amounts of faeces were insufficient. Instead, isolated strains from these foals were tested for the toxin A and B genes.

Isolation of *C. difficile*: Faecal material or the rectal swab was streaked on a selective agar medium, TCCFA-agar², containing cycloserine and cefoxitin, fructose, egg yolk and sodium taurocholate (to enhance growth of spores), as described previously (Båverud *et al.* 1997).

Identification of *C. difficile* and cytotoxin B assay: Typical colonies were identified by characteristic smell, colony morphology, Gram stain, biochemical tests and gas-liquid chromatography. *C. difficile* cytotoxin B assay was made on MRC-5 cells with faecal samples from mature horses and foals in Groups 2 and 4 (Båverud *et al.* 1997).

Culture for *Salmonella sp.*: Cultures were made from faecal samples of diarrhoeic horses, 40 in Group B and 70 in Group C, in 3 steps; pre-enrichment, selective enrichment and selective cultivation on agar plates (Anon 1991).

TABLE 4: Prevalence of *Clostridium difficile* in environmental samples

Source	No. samples	No. (%) positive samples for <i>C. difficile</i> by culture	No. positive isolates for toxins A and B by PCR
Outdoors			
Soil samples from studfarms	132	14 (11)	10
Soil samples from stables with mature horses	220	2 (1)	2
Soil samples from public parks, playgrounds, gardens and cultivated fields	246	9 (4)	6
Total	598	25 (4)	18
Indoors			
Environmental surface samples at the Faculty of Veterinary Medicine	100	3 (3)	2
Environmental surface samples at studfarms	120	3 (2)	2
Environmental surface samples in stables with mature horses	119	1 (1)	1
Floor samples in public places	95	0 (0)	-
Total	434	7 (2)	5

Environmental samples

Outdoor soil samples: Soil samples (15–20 ml) (n = 598) were taken from enclosed pastures and paddocks at 2 studfarms and 8 stables with only mature horses, from public parks, playgrounds, gardens and cultivated fields. The samples were packed in plastic tubes and cultured within 24 h. The soil was moistened with 0.9% sodium chloride (NaCl) for 30 mins, cultured as faecal samples and read after 2 and 5 days. Isolated strains were tested for toxin A and B genes by PCR.

Indoor surface samples: Samples (n = 434) were taken at the FVM (25 swabs at the Department of Small Animal Sciences and 75 at the Department of Large Animal Sciences), 2 riding schools, 6 private stables (15 swabs at each) and 2 studfarms (30 swabs at each). Surfaces sampled included floor, table, work surface, windowsill, floor drain, wall of stable, feed bowl and around watering device. Samples were also taken from floors in 19 public places (5 swabs at each); train station, restaurants, supermarkets, entrance halls and schools. Swabs were moistened with NaCl and 25 cm² in area was swabbed. The swabs were placed immediately in Amies medium with charcoal¹, cultured within 24 h (most within 6 h) on TCCFA-agar² and read after 2 and 5 days. Isolated strains were tested for toxin A and B genes by PCR.

Survival of *C. difficile* in equine faeces; an experimental study

Fresh *C. difficile*-free samples from the rectums of 3 healthy horses were mixed and inoculated with a *C. difficile* isolate (An767/94) isolated from a horse with antibiotic-associated diarrhoea (Gustafsson *et al.* 1997). Ten colonies of the strain were transferred to 100 ml of brain heart infusion (BHI)⁴ 37.0 g/l, with yeast extract⁴ 5.0 g/l, Resazurin solution 4.0 ml/l, Bacto agar⁴ 0.5 g/l, L-cysteine HCl⁵ 0.5 g/l and vitamin K1-haemin solution 10.0 ml/l, and incubated in an anaerobic chamber for 48 h at 37°C and for a further 5 days at 20°C for sporulation. The concentration of *C. difficile* in broth was estimated by total viable counts on FAA-agar³ with 5% defibrinated horse blood incubated at 37°C for 48 h. To 58 g of faeces, 3 ml of *C. difficile* broth was added and mixed well. The concentration was approximately 8 x 10³ colony-forming units/g faeces. The inoculated faeces were placed outdoors on the lawn in a villa garden in 2 places, one with and one without a roof, at ambient temperature over the year (between -15°C and +28°C).

Inoculated faeces were also kept indoors on a bench, in a glass jar without a lid, at room temperature. Once a month, about 1 g of the inoculated faeces was sampled in a plastic bag and moistened with 3 ml NaCl for 30 mins before cultivation on TCCFA-agar.

In vitro amplification of toxin A and toxin B gene fragments by PCR

Amplification of a 1217-bp toxin A gene fragment and a 1050-bp toxin B gene fragment was performed on strains isolated from environment and foals in *Groups 1* and *3* in a duplex PCR, according to McMillan *et al.* (1992), with some modifications. Primers for the toxin A gene were used a concentration of 0.2 µmol/l each and for the toxin B gene a concentration of 0.3 µmol/l each. Ten µl of bacterial DNA template was used in each reaction. The enzyme used was AmpliTaq DNA polymerase⁶, and the hot start before amplification was omitted. One negative and one positive strain for toxin B in a cell culture assay and for toxin A in an immunoassay (*C. difficile* toxin A test⁷) were used as negative and positive control strains.

Antimicrobial susceptibility testing

Minimum inhibitory concentrations (MIC) of ampicillin, bacitracin, benzylpenicillin, chloramphenicol, erythromycin, fusidic acid, metronidazole, rifampicin, trimethoprim/sulphamethoxazole and vancomycin for 52 isolated *C. difficile* strains were determined by the Etest⁸ on Wilkins-Chalgren agar⁷ supplemented with 5% defibrinated horse blood. The strains were isolated from 48 horses and 4 indoor environmental samples. A suspension of *C. difficile* was made in BHI without Bacto agar but with the supplements (described above) to McFarland standard 1 from a pure culture. The Etest was performed according to the manufacturer's instructions. The plates were incubated in an anaerobic chamber at 37°C for 48 h. The MIC value was read at the point of intersection between the inhibition ellipse edge and the Etest strip. Quality control strains used for the test included *Bacteroides fragilis* ATCC 25285 and *Bacteroides thetaiotaomicron* ATCC 29741. The MICs of the quality control strains were within recommended ranges (Anon 1997).

Results

Faecal specimens - mature horses

Clostridium difficile: In horses that developed acute colitis during antibiotic treatment (*Group B*), 18 of 43 horses (42%) were positive for *C. difficile* by culture and/or cytotoxin B assay of faeces (Table 1). Of these, 10 were positive for *C. difficile* by both culture and cytotoxin B assay, 6 by culture only and 2 by cytotoxin assay only. Twelve of the horses positive for *C. difficile* developed acute colitis at animal hospitals or had recently visited a clinic or an animal hospital.

Of the horses positive for *C. difficile*, all were treated with β-lactam antibiotics, alone or in combination with TMP/SDZ or gentamicin, except one that was treated with TMP/SDZ only.

Of *C. difficile*-positive horses with colitis and no antibiotic treatment (*Group C*), one developed diarrhoea at an animal hospital and 3 at their home stable without any prior contact with a clinic.

Salmonella sp. investigations: *Salmonella* Typhimurium, phage type 40, was isolated from one horse that was negative for *C. difficile* in *Group B*. All other horses tested for *Salmonella sp.* were negative.

Mortality: In *Group B*, 16 of 43 horses died or were subjected to euthanasia due to severe complications to the colitis. Of these, 2 were positive for *C. difficile* in culture and another 2 in both culture and cytotoxin B test. In *Group C*, 12 of 72 horses died or were subjected to euthanasia due to severe sequelae. Of these, one was positive for *C. difficile* in culture and cytotoxin B was not tested. For 29 horses in *Group C*, the outcome was not known.

Faecal specimens - foals

Clostridium difficile: In healthy untreated foals age 0–13 days (*Group 1*), faecal samples from 16 of 56 foals (29%) were positive for *C. difficile* by culture. Five of 14 tested isolates contained the toxin A and B genes as determined by PCR. In foals age 14–29 days, one of 37 (3%) was positive for *C. difficile* in culture and contained the toxin A and B genes (Table 2).

In foals with no diarrhoea, treated with erythromycin or gentamicin in combination with rifampicin (*Group 2*), *C. difficile* was isolated from 7 of 16 (44%) foals (Table 3). Of 15 tested faecal samples, 4 culture-positive foals proved positive in the cytotoxin B test.

In foals with no diarrhoea, treated with penicillin and/or TMP/SDZ (*Group 3*), 2 of 13 (15%) were positive for *C. difficile* by culture (Table 3). One isolate contained the toxin A and B genes.

In foals with diarrhoea (*Group 4*), 2 of 22 (9%) proved positive by culture for *C. difficile*. Both positive foals were hospitalised (Table 3). Toxins were not tested in *Group 4*.

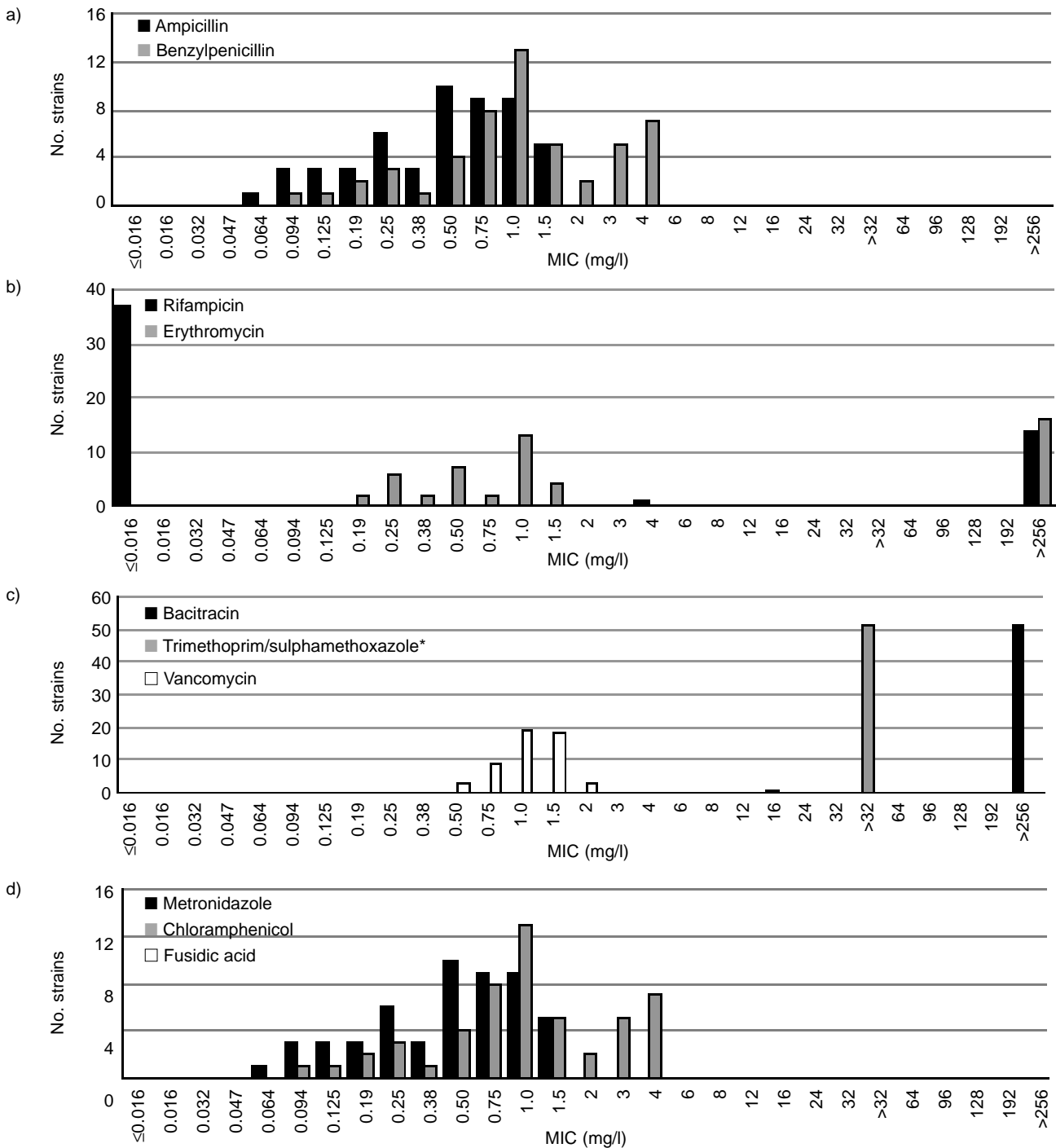
Environmental samples

Outdoor soil samples: *C. difficile* was isolated from 14 of 132 (11%) soil samples from enclosed pastures and paddocks at stud farms, but from only 2 of 220 (1%) samples from pastures and paddocks with mature horses ($P < 0.001$ by Chi-square analysis). In soil samples from public parks, playgrounds, gardens and cultivated fields, 9 of 246 (4%) samples were positive. The toxin A and B genes were demonstrated in 18 of the total 25 isolates from soil samples (Table 4).

Indoor surface samples: In environmental surface samples from the FVM, 3 of 100 (3%) were positive by culture for *C. difficile*. Of these 3, two were from 2 different floor drains at the Department of Small Animal Sciences and one was from a shoe sole of a veterinarian at the Department of Large Animal Sciences. *C. difficile* was isolated from 3 of 120 (2%) surface samples from studfarms and from one of 119 (1%) surface samples from stables with mature horses. The toxin A and B genes were demonstrated in 5 of the total 7 isolates from indoor surface samples. *C. difficile* was not isolated from any sample taken from floors in public places (Table 4).

Survival of C. difficile in equine faeces; an experimental study

In our experimental study of survival of *C. difficile* in inoculated equine faeces, the bacterium was found to survive for at least 4 years (not further tested) when kept at room temperature and outdoors at ambient temperature over the year.



Figs 1a-d: Distribution (no. strains) of MIC values (mg/l) for 52 *Clostridium difficile* strains of 10 antibiotics. c) One strain not tested for bacitracin; d) Six strains not tested for metronidazole. *When MIC value was above the range tested, the value for the next titration step (the value just above the range) was used.

Antimicrobial susceptibility testing

The distribution of MIC values for the antibiotics included in the study for the 52 *C. difficile* strains is presented in Figures 1a–d. All strains were resistant to trimethoprim/sulphamethoxazole (MIC > 32 mg/l) and bacitracin (MIC > 256 mg/l). All strains were classified as susceptible to metronidazole (MIC ≤ 4 mg/l), vancomycin (MIC ≤ 2 mg/l) and fusidic acid (MIC ≤ 0.75 mg/l).

Fourteen strains were uniformly resistant to erythromycin (MIC > 256 mg/l) and rifampicin (MIC > 256 mg/l). Four of these 14 strains also had higher MIC values to chloramphenicol (MIC = 12–32 mg/l). Three of the 14 strains were isolated from horses treated with erythromycin in combination with rifampicin at one animal hospital. All strains isolated from horses treated with other antibiotics at the same animal hospital were susceptible to erythromycin and rifampicin. At another animal hospital, however,

strains resistant to erythromycin and rifampicin were found in horses treated both with erythromycin and with β -lactam antibiotics.

Discussion

C. difficile in mature horses

The present investigation further confirms that *C. difficile* is associated with acute colitis in mature horses previously treated with antibiotics (Beier *et al.* 1994; Cosmetatos *et al.* 1994; Madewell *et al.* 1995; Gustafsson *et al.* 1997; Båverud *et al.* 1997, 1998). From the horses with antibiotic-associated diarrhoea, 42% of faecal samples were positive for *C. difficile* in culture and/or cytotoxin B. Notably, 28% (12/43) of the mature horses with antibiotic-associated diarrhoea were positive in the cytotoxin B test, which is consistent with the results in human medicine (Bartlett 1990). *C. difficile* was not isolated from faecal samples of horses without signs of enteric disorders and with no history of previous treatment with antibiotics, nor from horses with colic. In horses with colitis and no history of previous antimicrobial use, *C. difficile* was isolated from 4 of 72 (6%) tested horses. Three of those 4 horses were tested and found negative for cytotoxin B. In other studies, *C. difficile* was also isolated from diarrhoeic horses without previous antimicrobial therapy (Beier *et al.* 1994; Cosmetatos *et al.* 1994).

Faecal samples from 2 horses with antibiotic-associated diarrhoea were culture-negative but toxin-positive for *C. difficile*. The lack of anaerobic storage of faeces probably reduced the apparent incidence of *C. difficile* isolation (Weese *et al.* 2000a).

C. difficile in foals

A novel interesting finding of this study was that about 30% of healthy foals during the first 13 days *post partum* proved positive in culture for *C. difficile*, whereas in the age group 1–6 months, all 133 foals were negative. This is analogous with human infants and canine neonates, who also have a high carrier rate (Merida *et al.* 1986; Tullus *et al.* 1989; Perrin *et al.* 1993). It was demonstrated by PCR that strains from healthy foals can serve as a potential reservoir of toxin-producing *C. difficile*.

However, 7/16 (44%) nondiarrhoeic foals age 1–3 months also carried *C. difficile* when treated with antibiotics. Erythromycin, or gentamicin in combination with rifampicin, seemed to select for carriage of *C. difficile* in those foals. Combinations of antibiotics have a higher risk of inducing colonisation of *C. difficile* and the development of the disease in man (Tabaqchali and Jumaa 1995). Very young foals, age 0–13 days, were carriers of *C. difficile* irrespective of whether they were treated with antibiotics.

In recent years, *C. difficile* has been associated with diarrhoea in foals (Jones *et al.* 1987, 1988a,b; Magdesian *et al.* 1999; Weese *et al.* 2001). We were unable to demonstrate this, as only 2 of 22 (9%) of the diarrhoeic foals were positive for *C. difficile* in culture and both were treated with at least 3 different antibiotics before the onset of diarrhoea. The results of the present study show that, generally, the common occurrence of *C. difficile* in very young foals is not associated with disease.

Occurrence and survival of *C. difficile* in the environment

At studfarms, *C. difficile* was isolated from 11% of soil samples taken from paddocks and enclosed pastures but from only 1% of soil samples from stables with mature horses ($P < 0.001$). This

difference may be related to the high excretion rate of the bacterium in the faeces of neonatal foals compared with mature horses. The isolation frequency from public parks and playgrounds varied between different sampling places. There was a tendency towards more positive soil samples in connection with water-filled ditches. PCR showed that environmental isolates of *C. difficile* are potential toxin producers.

Notably, the indoor environmental surface samples from the Department of Large Animal Sciences, Faculty of Veterinary Medicine, were all negative for *C. difficile*, except one sample from a veterinarian's shoe sole. The spores of *C. difficile* are known to persist for long periods (Tabaqchali and Jumaa 1995). Since there were horses with positive faecal samples at the clinic during recent years, it was expected that some isolates would be found. In Canada and England, *C. difficile* was found in 6.3 and 16.7%, respectively, of the indoor environment samples in a veterinary hospital (Al Saif and Brazier 1996; Weese *et al.* 2000b). In this study, 3% of the samples at the Swedish Faculty of Veterinary Medicine were positive.

The present study demonstrates that *C. difficile* spores survive in inoculated equine faeces for at least 4 years, both indoors and outdoors. The outdoor-placed faeces had decomposed after about a year, but culturable *C. difficile* was still present in the soil. However, in its vegetative form, the bacteria has poor survival in aerobically-stored faecal samples (Weese *et al.* 2000a). Furthermore, our experience is that *C. difficile* can be isolated and cytotoxin B demonstrated from faecal samples stored at -20°C for at least 4 years.

Antimicrobial susceptibility testing

Antimicrobial drugs such as metronidazole and vancomycin are effective in the treatment of *C. difficile*-associated diarrhoea in man (Kelly *et al.* 1994; Job and Jacobs 1997). Currently, metronidazole is recommended as the first-line treatment for *C. difficile*-associated diarrhoea (Job and Jacobs 1997). However, metronidazole-resistant strains of *C. difficile* have been isolated from human subjects (Kelly *et al.* 1994) and, recently, also from American horses (Jang *et al.* 1997). There was no resistance to metronidazole or vancomycin among strains isolated from Swedish horses, although the MIC value (2–4 mg/l) of metronidazole was relatively high for 4 strains. Bacitracin has been suggested as a suitable antibiotic for treatment of acute idiopathic colitis in horses (Staempfli *et al.* 1992). All tested strains in this study were classified as resistant to bacitracin (MIC > 256 mg/l).

For erythromycin and rifampicin, a distinct group of 14 *C. difficile* strains with high MIC values was distinguished. These strains were considered resistant to these agents. All strains isolated at one animal hospital were resistant irrespective of agents used, indicating that *C. difficile* was a nosocomial problem at this animal hospital.

In conclusion, *C. difficile* is associated with acute colitis in mature horses previously treated with antibiotics. There is a high frequency of carriers in apparently healthy neonatal foals and in nondiarrhoeic foals treated with antibiotics. Isolated strains were susceptible to metronidazole and vancomycin. The occurrence of *C. difficile* was more common in soil samples from studfarms than from farms with mature horses. The presence of toxin A and toxin B genes in isolates of *C. difficile*, demonstrated by PCR, in both healthy foals and the environment, shows that these strains can serve as a potential reservoir of toxigenic *C. difficile*. The bacteria survived for at least 4 years in nature.

Manufacturers' addresses

- ¹Venturi Transystem, Copan, Brescia, Italy.
²National Veterinary Institute, Uppsala, Sweden.
³LabM, Bury, Lancashire, UK.
⁴Difco, Detroit, Michigan, USA.
⁵Sigma, St. Louis, Missouri, USA.
⁶Applied Biosystems, Foster City, California, USA.
⁷Oxoid, Unipath Ltd, Basingstoke, Hampshire, UK.
⁸AB Biodisk, Solna, Sweden.

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