Molecular characterization of *Clostridium difficile* isolates from horses in an intensive care unit and association of disease severity with strain type

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**Objective**—To determine molecular characteristics, antimicrobial susceptibility, and toxigenicity of *Clostridium difficile* isolates from horses in an intensive care unit and evaluate associations among severity of clinical disease with specific strains of *C difficile*.

**Design**—Prospective study.

**Animals**—130 horses.

**Procedures**—Feces were collected from horses admitted for acute gastrointestinal tract disease with loose feces and submitted for microbial culture and immunohistoassay for toxin production. Polymerase chain reaction assays were performed on isolates for toxins A and B genes and strain identification.

**Results**—Isolates were grouped into 3 strains (A, B, and C) on the basis of molecular banding patterns. Toxins A and B gene sequences were detected in 93%, 95%, and 73% of isolates of strains A, B, and C, respectively. Results of fecal immunoassays for toxin A were positive in 40%, 63%, and 16% of horses with strains A, B, and C, respectively. Isolates in strain B were resistant to metronidazole. Horses infected with strain B were 10 times as likely to have been treated with metronidazole prior to the onset of diarrhea as horses infected with other strains. Duration from onset of diarrhea to discharge (among survivors) was longer, systemic inflammatory response syndromes were more pronounced, and mortality rate was higher in horses infected with strain B than those infected with strains A and C combined.

**Conclusions and Clinical Relevance**—Horses may be infected with a number of heterogeneous isolates of *C difficile*. Results indicated that toxigenicity and antimicrobial susceptibility of isolates vary and that metronidazole-resistant strains may be associated with severe disease.

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*Clostridium difficile* has been associated with diarrhea and colitis in horses and foals as well as in humans, rodents, rabbits, swine, and a number of other species. In humans, the clinical state associated with colonization by *C difficile* is notoriously unpredictable, varying from asymptomatic shedding to severe disease, with patients often having SIRS, septic shock, or acute respiratory distress syndrome. In humans, the largest risk factor for development of CDAD is the administration of antimicrobials, and the same association has been made in horses. Additional risk factors in humans include advancing age, immunosuppression, renal disease, and administration of chemotherapeutics or proton pump inhibitors such as omeprazole. In neonatal infants and foals, however, *C difficile* can be a primary pathogen. Colonization may also develop asymptptomatically and commonly develops in group-housed infants. Horses with *C difficile*-associated diarrhea are reported to have a higher mortality rate than those with diarrhea that are *C difficile* negative. The pathogenesis of the disease is attributable to a number of virulence factors, including large enterotoxins such as toxins A (enterotoxin) and B (cytotoxin). Adenosine diphosphate-ribosyltransferase (binary toxin) has also been detected in equine isolates of *C difficile*; its role in the pathogenesis of disease has not been established.

A large number of different genetic strains or clones of *C difficile* have been associated with diarrhea and colitis in human patients, and an association between strain type and severity of clinical disease has been established. Strain discrimination of isolates from humans has been reported by use of several techniques, including random amplification of polymorphic DNA (AP-PCR assay), pulsed-field gel electrophoresis, serotyping, SDS-PAGE, immunoblotting, ribotyping, and restriction enzyme analysis techniques. Molecular fingerprinting of equine isolates has also been reported for strain identification. However, to the authors’ knowledge, a large-scale evaluation of equine isolates has not been reported. Additionally, to the authors’ knowledge, studies associating clinical virulence with equine strains of *C difficile* have not been published.

The antimicrobial susceptibility patterns of isolates of *C difficile* from humans predictably, with few exceptions, include susceptibility to metronidazole and vancomycin. Resistance to metronidazole has been rarely reported among isolates from humans; however, it is quite common among equine isolates studied at our hospital. The purposes of the study reported here...
here to determine molecular characteristics, antimicrobial susceptibility, and toxigenicity of C. difficile isolates from horses in an ICU with enteric disease were studied. Horses were chosen on the basis of admission to the enteric ICU for gastrointestinal tract disease with loose stools or development of loose stools while hospitalized. Horses in which results of bacteriologic culture of feces were positive for C. difficile were considered to have C. difficile-associated diarrhea. Bacteriologic culture of feces for C. difficile was performed by use of selective medium containing cycloserine, cefoxitin, and fructose agar. Isolates were positively identified as C. difficile via colonial morphology on the basis of selective medium, gram-staining characteristics, lack of aerotolerance, and unique fatty acid production.\(^3\)\(^4\)\(^5\) Toxigenicity of isolates was assessed by use of 1 of 2 commercial immunoassays\(^6\)\(^7\) for toxin A and PCR assay for toxins A and B gene sequences, as described.\(^8\)\(^9\) Two oligonucleotide primer pairs designed to amplify toxins A and B gene sequences were used. Molecular fingerprinting of C. difficile isolates was performed by use of an AP-PCR assay with a 15-mer (33G plus C) oligonucleotide for random amplification of polymorphic DNA, as previously reported.\(^10\)\(^11\)\(^12\)\(^13\) Briefly, the arbitrary primer used was PG-05, a 15-mer oligodeoxynucleotide (5’-AGGCCCGACTTGAAC-3’).\(^14\) PG-05 is the sequence found in the coding region of the human interleukin-6 gene. Isolation of DNA was performed by lysozyme and detergent lysis, followed by incubation with a nontoxic proteinase and a series of phenol, chloroform, and isooctyl alcohol extractions prior to alcohol precipitation of nucleic acids. The DNA concentration was determined via fluorometry. The PCR assay was performed in a 50-µL reaction mixture containing PCR assay buffer, 500 pmol of primer PG-05, 200 µL each of deoxynucleotide triphosphate and 0.001% gelatin, 2.5 units of recombinant Taq polymerase,\(^15\) and 25 ng of DNA template. Forty-five cycles of amplification were performed as follows: denaturation at 92°C for 1 minute, annealing at 36°C for 1 minute, and extension at 72°C for 2 minutes. With each cycle, the annealing temperature was increased by 0.2°C. Ten-milliliter aliquots of the reaction mixture were separated electrophoretically in 1.5% agarose gels in TRIS-acetate-EDTA buffer. A 123-bp DNA ladder was included as a size marker in all gels, and 1 isolate was used as an internal control from gel to gel. Gels were run at 110 V for 130 minutes. Gels were stained in ethidium bromide solutions for 30 minutes, destained in deionized water, and photographed under ultraviolet light. The AP-PCR assay types were differentiated by visualization of the gels. This technique has good correlation with densitometry in differentiation of genetic types of C. difficile.\(^16\)

Antimicrobial susceptibility testing was performed by use of the E-test method, as described.\(^17\)\(^18\) Briefly, the E test was performed by use of Brucella agar plates inoculated to confluence with a broth culture of C. difficile of sufficient density to match a 0.5 McFarland turbidity standard. The MIC value was determined to be the intercept of the inhibition ellipse on the E-test scale and imprinted on the strip. Isolates were considered susceptible to metronidazole at MIC values ≤ 8 µg/mL and to vancomycin at values ≤ 4 µg/mL. These cutoff values were based on values from the Clinical and Laboratory Standards Institute.

**Materials and Methods**

Isolates from horses evaluated at the University of California Veterinary Medical Teaching Hospital large-animal ICU with acute enteric disease were studied. Horses were chosen on the basis of admission to the enteric ICU for gastrointestinal tract disease with loose stools or development of loose stools while hospitalized. Horses in which results of bacteriologic culture of feces were positive for C. difficile were considered to have C. difficile-associated diarrhea. Bacteriologic culture of feces for C. difficile was performed by use of selective medium containing cycloserine, cefoxitin, and fructose agar. Isolates were positively identified as C. difficile via colonial morphology on the basis of selective medium, gram-staining characteristics, lack of aerotolerance, and unique fatty acid production.\(^3\)\(^4\)\(^5\) Toxigenicity of isolates was assessed by use of 1 of 2 commercial immunoassays\(^6\)\(^7\) for toxin A and PCR assay for toxins A and B gene sequences, as described.\(^8\)\(^9\) Two oligonucleotide primer pairs designed to amplify toxins A and B gene sequences were used. Molecular fingerprinting of C. difficile isolates was performed by use of an AP-PCR assay with a 15-mer (33G plus C) oligonucleotide for random amplification of polymorphic DNA, as previously reported.\(^10\)\(^11\)\(^12\)\(^13\) Briefly, the arbitrary primer used was PG-05, a 15-mer oligodeoxynucleotide (5’-AGGCCCGACTTGAAC-3’).\(^14\) PG-05 is the sequence found in the coding region of the human interleukin-6 gene. Isolation of DNA was performed by lysozyme and detergent lysis, followed by incubation with a nontoxic proteinase and a series of phenol, chloroform, and isooctyl alcohol extractions prior to alcohol precipitation of nucleic acids. The DNA concentration was determined via fluorometry. The PCR assay was performed in a 50-µL reaction mixture containing PCR assay buffer, 500 pmol of primer PG-05, 200 µL each of deoxynucleotide triphosphate and 0.001% gelatin, 2.5 units of recombinant Taq polymerase,\(^15\) and 25 ng of DNA template. Forty-five cycles of amplification were performed as follows: denaturation at 92°C for 1 minute, annealing at 36°C for 1 minute, and extension at 72°C for 2 minutes. With each cycle, the annealing temperature was increased by 0.2°C. Ten-milliliter aliquots of the reaction mixture were separated electrophoretically in 1.5% agarose gels in TRIS-acetate-EDTA buffer. A 123-bp DNA ladder was included as a size marker in all gels, and 1 isolate was used as an internal control from gel to gel. Gels were run at 110 V for 130 minutes. Gels were stained in ethidium bromide solutions for 30 minutes, destained in deionized water, and photographed under ultraviolet light. The AP-PCR assay types were differentiated by visualization of the gels. This technique has good correlation with densitometry in differentiation of genetic types of C. difficile.\(^16\)

Statistical analysis—Statistical analysis consisted of descriptive statistics for vital signs as markers of SIRS, including rectal temperature, heart rate, and respiratory rate at admission. Similar analyses were performed for duration of hospitalization, duration from development of diarrhea to admission (for horses admitted with diarrhea) or duration from admission to development of diarrhea (for horses developing diarrhea after admission), duration of diarrhea, as well as for the duration from onset of diarrhea to discharge. A Mann-Whitney U statistic was used to evaluate differences in vital signs as markers of SIRS, risk of death, duration of diarrhea, duration from admission to development of diarrhea, and duration of hospitalization among horses with various isolate types. A Fisher exact test was used to compare survival among horses infected with various isolate types. A value of \(P \leq 0.05\) was considered significant.

**Results**

Feces were obtained from 130 horses admitted to a veterinary teaching hospital large-animal ICU with enteric disease for microbial culture for C. difficile. Most (122/130) of these horses were admitted to the ICU for a clinical diagnosis of acute diarrhea or colitis, whereas 8 horses were admitted with signs of abdominal pain. Mean ± SD age of horses was 7.7 ± 6 (median, 6 years; range, 1 to 29 years). The population included 66 mares, 48 geldings, and 16 stallions. Eighteen breeds were represented, although Thoroughbreds (n = 53) and Quarter Horses (25) were the largest breed groups.

Results of molecular fingerprinting via AP-PCR assay indicated 2 distinct strain types designated strains A and B, which were isolated from 15 and 24 horses, respectively. The remaining 91 horses had isolates that were heterogeneous and difficult to categorize into discrete groups on the basis of DNA banding patterns. These diverse isolates were grouped and called strain C for comparison purposes.

Results of PCR assay for toxins A and B gene sequences indicated that both toxins A and B sequences were present in 93%, 95%, and 73% of isolates of strain types A, B, and C, respectively. Both A and B genes were missing, consistent with nontoxicogenic strains, from 7%, 0%, and 20% of strain types A, B, and C, respectively. Isolates that were A+/B− (positive for toxin A gene but negative for toxin B gene) represented 5% (1/21) of isolates in strain B and 4% (5/88) of isolates in strain C. One percent of isolates (1/88) in strain C was toxin A−/B+ (negative for toxin A gene but positive for toxin B gene). Fecal immunoassay results for toxin A were positive for 40%, 63%, and 16% of horses with strains A, B, and C, respectively, compared with 93%, 100%, and 79% as detected via PCR assay.

Within strain A, 93% of isolates were susceptible to metronidazole, whereas none of the isolates within strain B were susceptible to metronidazole. In strain C, 96% of the isolates were susceptible to metronidazole. Isolates in strain B were significantly \((P < 0.001)\) more likely to be resistant to metronidazole than isolates in strains A or C. All isolates of all strain types were susceptible to vancomycin (≤ 2 µg/mL).

Metronidazole administration prior to development of diarrhea was also evaluated. None of the horses with strain A had been treated with metronidazole before the onset of diarrhea. Only 2% of horses with strain C had received metronidazole, whereas 58% of...
horses with strain B (100% resistant to metronidazole) had been treated with metronidazole. A horse infected with strain B was 10 times as likely to have been treated with metronidazole prior to the onset of diarrhea as horses infected with either of the other strains (relative risk, 10; 95% confidence interval, 5.4 to 18.6; \( P < 0.001 \)); relative risk equals the ratio of the probability of developing an outcome [in this case, history of metronidazole use] among those receiving the treatment of interest or exposed to a risk factor [in this case, infection with strain B], compared with the probability of developing the outcome if the risk factor or intervention is not present [ie, the ratio of risk of having been treated with metronidazole in group B horses (infected with strain B) to the risk of having been treated with metronidazole in group A or C horses]).

For purposes of evaluating strain effect on clinical severity, strains A and C were grouped (A and C combined) and compared with strain B because of the small number of horses with strain A as well as the distinct differences in metronidazole susceptibility between these strains (96% vs 0% of susceptible isolates). There was no significant difference between strains in the duration from admission to development of diarrhea or duration of hospitalization, but there was a significant difference for the duration from the onset of diarrhea to discharge among survivors in which the duration was longer in horses with strain B than in horses with strains A and C combined (Table 1).

Horses infected with strain B (metronidazole-resistant strain) had more severe SIRS, as evidenced by pyrexia and tachycardia, than horses infected with other strains of *C difficile*. Mean \( \pm \) SD rectal temperature in horses with strains A and C combined (38.3 \( \pm \) 0.7°C [101.1 \( \pm \) 1.3°F]; median, 38.3°C [101°F]) was significantly (\( P < 0.05 \)) different from rectal temperature in horses with strain B (38.8 \( \pm \) 0.8°C [101.8 \( \pm \) 1.4°F]; median, 38.7°C [101.6°F]). Mean \( \pm \) SD heart rate in horses with strains A and C combined (54 \( \pm \) 17 beats/min [median, 48 beats/min]) was significantly (\( P < 0.05 \)) different from heart rate in horses with strain B (65 \( \pm \) 20 beats/min [median, 56 beats/min]). Mean \( \pm \) SD respiratory rate in horses with strains A and C combined was not significantly different from mean \( \pm \) SD respiratory rate in horses with strain B; horses in both groups had mild tachypnea (strains A and C combined, 26 \( \pm \) 15 breaths/min [median, 24 breaths/min]; strain B, 31 \( \pm \) 16 breaths/min [median, 28 breaths/min]). There was a significant (\( P = 0.05 \)) difference in mortality rate among horses in the 2 groups; horses with strains A and C combined had a mortality rate of 27%, whereas 50% of horses with strain B died or were euthanized. The relative risk of death or euthanasia was 0.54 (95% confidence interval, 0.32 to 0.91) in horses with strains A and C combined, compared with horses with strain B.

**Discussion**

Results of the study reported here indicated that heterogeneity existed among isolates of *C difficile* associated with acute diarrhea or enterocolitis in horses. This variability was evident on the basis of molecular fingerprinting as well as antimicrobial susceptibility. A similar degree of heterogeneity also exists among *C difficile* isolates from human patients, even in outbreak situations. Two major molecular types were identified in the study by Samore et al, with multiple additional subtypes.

Resistance to metronidazole is not common among *C difficile* isolates in human patients, although it has been recently recognized in 6.3% of 415 isolates. On the other hand, metronidazole resistance is common among certain strains of *C difficile* from horses. As with isolates from human patients, metronidazole-resistant isolates have clonal heterogeneity, although most of the resistant isolates in our study were of 1 specific strain type. According to results of our study, treatment with metronidazole predisposed horses to colonization with resistant strains among horses that developed diarrhea associated with *C difficile*. In addition, it has been suggested that use of metronidazole be restricted to patients in which anaerobic antimicrobials are specifically indicated; this recommendation was made because of the compromising effects of metronidazole on the normal intestinal flora, which is comprised largely of anaerobes.

Metronidazole resistance has implications for treatment of *C difficile* infections in horses because it is the treatment of choice in human patients with gastrointestinal tract disease attributable to *C difficile*. Metronidazole has been suggested as the recommended treatment for horses with CDAD as well; however, results of our study and other studies indicate that antimicrobial susceptibility testing of isolates is warranted. Intermediate resistance to vancomycin (4 to 16 \( \mu g/mL \)) has been reported in 3.1% of isolates from humans, although all isolates tested in our study were susceptible to vancomycin at \( \leq 2 \mu g/mL \). This finding has implications for future treatment protocols, although we believe that the use of vancomycin should be restricted and used only when metronidazole resistance is documented or suspected. The MIC breakpoints are set for isolates causing systemic infections and are based on antimicrobial concentrations in serum. In cases of gastrointestinal tract disease associ-

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<tr>
<th>Parameter</th>
<th>Strain</th>
<th>( P \text{ value} )</th>
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<tr>
<td>Duration from admission to development of diarrhea (d)</td>
<td>A and C combined</td>
<td>5.4 ± 13.3 (0)</td>
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<tr>
<td>Duration of hospitalization (d)</td>
<td>7.9 ± 5.7 (7)</td>
<td>16.2 ± 19.7 (10.5)</td>
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<tr>
<td>Duration from onset of diarrhea to discharge among survivors (d)</td>
<td>7.9 ± 6.6 (7)</td>
<td>18.5 ± 17.8 (14)</td>
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Table 1—Mean \( \pm \) SD (median) values for clinical parameters among horses with *Clostridium difficile*-associated diarrhea in an ICU with metronidazole-susceptible (A and C combined) and -resistant (B) strains of *C difficile*. 

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ated with *C. difficile* infection, concentrations in the colon may be more appropriate than serum concentrations for determining MIC breakpoints. However, to the authors’ knowledge, concentrations of metronidazole in gastrointestinal tracts of horses have not been explored.

To the authors’ knowledge, this is the first report to describe clinical findings in adult horses with *C. difficile* infection and their associations with individual strain types. Metronidazole-resistant isolates of *C. difficile* are associated with more severe clinical disease than are metronidazole-susceptible isolates, as evidenced by a more significant SIRS response, higher mortality rate, longer duration of hospitalization, and longer duration from onset of diarrhea to discharge. A similar association between strain type and toxigenicity has been described in human patients with CDAD. Results of 1 study in humans indicate that certain specific isolate groups are associated with a 1.5-fold higher likelihood of symptomatic disease than other isolate groups. Severity of clinical disease also varies with strain type in human patients; specific strain types have increased virulence and enhanced transmissibility during outbreaks. This variability in pathogenicity may be attributable to differences in mucosal adhesion, amount of toxin produced, or other virulence factors.

Typing of isolates is epidemiologically useful. Because of the marked variability among equine isolates, strain differentiation is required before assuming patient-to-patient transmission among temporally or spatially related cases. The source of *C. difficile* infection in human patients is often the environment or health workers within the hospital and is reflected in the wide diversity of strain types infecting patients concurrently. Techniques used for typing of *C. difficile* isolates include those based on phenotypic characteristics such as bacteriocin and bacteriophage susceptibility, electrophoretic protein patterns, and immunologic markers. Methods targeting genetic markers are more discriminatory than these methods, including restriction endonuclease analysis, ribotyping, pulse-field gel electrophoresis, and immunoblot analysis. The AP-PCR assay used in our study has advantages of being a rapid and sensitive typing scheme and is more time efficient than other techniques. The AP-PCR assay was more discriminatory than immunoblotting, and results were similar to that of restriction endonuclease analysis in 1 study. An additional random primer has been used for differentiation of *C. difficile* isolates: a 19-mer designated T-7. This primer was useful for differentiating isolates into major types; however, the PG-05 primer differentiates to a much higher level of discrimination than the T7 primer. In 1 study, a 2-level approach for differentiating isolates was evaluated, the T-7 primer was used to differentiate group strains into major types, and a second level of differentiation was used as the PG-05 primer. The T-7 primer produces DNA profiles with 6 to 10 bands, helping to identify isolates into major groups, whereas the PG-05 primer yields 7 to 16 bands, leading to more unique DNA fingerprinting. We chose to use only the PG-05 primer in our study because in the report by Tang et al, there were no cases in which the T-7 primer further discriminated isolates grouped in the same type as detected via the AP-PCR assay with PG-05. The T-7 primer therefore offered no advantages over use of the PG-05 primer alone. Typing via AP-PCR assay will also contribute to the study of horses with relapsing diarrhea, whether a horse is reinfected with the same or different strain, and whether horses become subclinical shedders after resolution of diarrhea. It is also a means of identifying cross-infection between patients and contamination from environmental surfaces and hospital personnel. Reported shortcomings of the AP-PCR assay are its sensitivities to changes in primer concentration, DNA concentration, brand of thermal cycler used, and DNA polymerase. Therefore, these factors must be considered when performing AP-PCR assays.

The finding of a discrepancy between PCR assay results for toxin A gene sequences and those of fecal immunoassays for toxin A is consistent with findings reported in other studies. Toxic production may be discordant with a genetic type of isolate. Other reasons may include insensitivity of the immunoassay, lack of in vivo production of toxins (silent genes), production of defective toxin A, or a low level of toxin gene transcription. Premature stop codons and mutations may also be responsible for these differences. Deletions in the repeating sequences (clostridial repetitive oligopeptide region) of the toxin A gene of toxin A-negative, toxin B-positive *C. difficile* strains have also been reported. Such isolates apparently do not produce biologically active toxin A, although some portions of the toxin A gene are present.

To the authors’ knowledge, the study reported here is the first to describe a number of equine isolates of *C. difficile* that contain gene sequences for only 1 of the 2 major *C. difficile* toxins, either enterotoxin (toxin A) or cytotoxin (toxin B). To the authors’ knowledge, this is also the first report of A+/B– (positive for toxin A gene but negative for toxin B gene) strains of *C. difficile* in horses. We have previously reported on 1 isolate from a foal that was A–/B+ (negative for toxin A gene but positive for toxin B gene). In the past, human isolates were considered to be pathogenic only if they contained both toxin A and B genes, which were believed to act synergistically. However, it has been recognized that isolates producing only one of these toxins can be pathogenic. Other virulence factors, such as binary toxin, may contribute to development of CDAD.

Variability in the presence or absence of toxin A and B gene sequences within specific groups of isolates represents genetic diversity within a single strain type. This was detected in our study, reflected by the fact that not all isolates within each group contained both gene sequences. This finding has also been reported in *C. difficile* isolates from human and environmental sources. No correlation was detected between molecular type and toxin production by use of this typing system.

The study reported here draws attention to the developing problem of antimicrobial resistance. *Clostridium difficile* isolates should not be considered uniformly susceptible to metronidazole or even to vancomycin. Results of other studies indicate widespread resistance to bacitracin. Further study is required to define additional pre-
disposing factors for *C. difficile* infection in horses, particularly those that are not susceptible to metronidazole. Presently, judicious and careful use of metronidazole in particular those that are not susceptible to metronidazole.

References