Test Selection and Interpretation in the Diagnosis of *Clostridium difficile*–Associated Colitis

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*Clostridium difficile* survives only for a short period in routinely stored fecal samples, although its toxins are quite stable. If there is to be a delay from sample collection to processing, *C. difficile* may not be cultured but *C. difficile* toxins should be present. Therefore, toxin-positive, culture-negative results should be considered diagnostic for *C. difficile*-associated colitis. Dept. of Clinical Studies (Weese and Staempfli) and Dept. of Pathobiology (Prescott), Ontario Veterinary College, University of Guelph, Guelph, ON, Canada N1G 2W1. © 1999 AAEP.

1. Introduction

*Clostridium difficile* is increasingly recognized in cases of colitis in adult horses and foals. In the past year at the Ontario Veterinary College, 25% of cases of colitis (n = 28) in adults and 30% in foals (n = 20) were associated with *C. difficile*. Currently, selective bacterial culture and *C. difficile* toxin assays are the main methods used to diagnose *C. difficile*-associated colitis. This anaerobic bacterium exerts its effects through the production of two toxins. Selective culture media and anaerobic conditions are required for the isolation of *C. difficile*. As a result, all diagnostic laboratories may not offer this service. Bacterial culture is also time-consuming, with a minimum 48-hour turnaround time. Further isolation of the organism without demonstration of toxins in the feces is not diagnostic because approximately 25% of *C. difficile* strains lack the ability to produce toxins. The significance of isolation of toxigenic strains of *C. difficile* in the absence of detectable toxins in the feces is unclear.

Detection of toxins in the feces, with or without isolation of the organism, is considered diagnostic. When bacterial culture and toxin assays are used together, a number of cases with discrepant results can be encountered. Culture-positive/toxin-negative results can be attributed to infection with a nontoxigenic strain. In such cases, isolation of *C. difficile* is not clinically relevant because these strains are not thought to be able to cause disease. Poor survival of *C. difficile* toxins in feces and toxin assay error have been suggested as alternative reasons. Culture-negative/toxin-positive results can be attributed to poor survival of the organism or false-positive toxin assay results. Commercially available ELISA test kits have good sensitivities and specificities for the detection of *C. difficile* toxins, reducing the chances of false toxin assay results. The survival characteristics of *C. difficile* and its toxins may be involved in discrepant results and need to be studied. Little information is available on the survival characteristics of this pathogen and its toxins. This study was designed to evaluate the survival of
C. difficile and its toxins in equine feces. This information is important for the equine practitioner with regard to test selection and interpretation in cases of colitis.

2. Materials and Methods

A. Experiment 1

Standard C. difficile suspension was inoculated into 8 g of C. difficile-free equine feces. Forty-nine samples from 14 C. difficile isolates were prepared and stored aerobically at 4°C. Twenty-three samples were prepared and stored anaerobically at 4°C. Samples were inoculated daily onto a selective and differential culture medium and incubated anaerobically at 37°C for 48 hours. Colonies were identified as C. difficile based on characteristic shape, color change in the medium surrounding the colony, gram stain and production of l-proline aminopeptidase.

B. Experiment 2

Nine isolates were inoculated into cooked meat broth medium and incubated anaerobically at 37°C until C. difficile toxins were detected in the supernatant via a commercially available ELISA. 10 ml of supernatant was then added to 8 g of C. difficile-free equine feces. The samples were stored aerobically at 4°C and tested regularly for the presence of C. difficile toxins.

C. Experiment 3

A small amount of feces from 16 C. difficile-inoculated fecal samples was collected on a sterile swab and plunged into a commercial anaerobic transport medium. The transport medium and fecal sample were stored aerobically at 4°C for 72 hours then inoculated onto C. difficile medium as described above.

3. Results

A. Experiment 1

C. difficile was recovered for 2.6 ± 2.8 days (mean ± SD, range 0–12 d). No growth was obtained from 24% (12/49) after 24 hours of aerobic storage. This increased to 33% and 61% after 48 and 72 hours, respectively. Growth was obtained from all (26/26) samples stored under anaerobic conditions for a minimum of 16 days. There was significantly higher growth from anaerobically stored samples by 24 hours.

B. Experiment 2

C. difficile toxins were detected in all inoculated samples for a minimum of 60 days.

C. Experiment 3

After 72 hours, C. difficile was cultured from all 16 samples stored in the anaerobic transport medium, but from only 2/16 stored aerobically. When growth of C. difficile did occur, it was significantly greater in samples stored in the transport medium.

4. Discussion

Because of its emergence as a significant pathogen in cases of equine colitis, practitioners should consider C. difficile as a potential cause in cases of colitis and submit appropriate samples to diagnostic laboratories that will perform selective culture and toxin testing. The survival characteristics of C. difficile and its toxins must be considered when submitting and interpreting laboratory test results. This may be especially relevant when samples are delayed from the time of collection to the time of processing, such as would be expected when specimens are submitted to external laboratories. C. difficile does not survive for an extended period in equine feces stored under refrigeration. It is apparent that poor aerotolerance is the reason for this. In contrast, C. difficile toxins remain detectable in inoculated fecal samples for the duration of the study period (60 days).

Positive culture results can support a positive toxin assay; however, culture-positive/toxin-negative results may occur because of nontoxicogenic strains not associated with disease. This study shows that C. difficile toxins are stable in equine feces and degradation of toxins should not be considered the cause of culture-positive/toxin-negative results. Furthermore, the results of this study suggest that the use of an anaerobic transport medium may be beneficial in situations in which there will be a delay before sample processing takes place. A prospective clinical trial is required to confirm this in a field situation.

Based on these results, we recommend that equine practitioners submit fecal samples for C. difficile toxin assays in all cases of colitis. Toxin assays are recommended because positive results can be considered diagnostic for C. difficile-associated colitis, delays in sample submission will not affect results and good commercial ELISA kits are now available that are rapid, easy to use and cost-effective. Samples can also be submitted for selective culture, but the diagnostic value of culture alone is limited. If culture of the organism is desired, it may be beneficial to submit fecal samples in an anaerobic transport medium.

Before submission of samples, practitioners should contact local diagnostic laboratories to ensure that the appropriate assays are available. C. difficile is an important pathogen in humans, so it may be possible to get testing performed at human medical laboratories if local veterinary laboratories do not offer the service. Fresh fecal samples are recommended for toxin assays and are essential if culture is to be successful. Ideal samples are those that are passed during examination or collected manually per rectum. Fecal samples should be placed in appropriate containers that are filled as much as possible, limiting the amount of air that is with the
samples. If culture is going to be requested, then a commercial anaerobic transport medium can be used along with the standard sample. When a semisolid agar anaerobic transport medium\(^d\) is used, a sterile swab should be inserted in the fecal sample, then plunged into the transport medium. The tip of the swab should be broken off and the lid sealed immediately. Samples should be stored at refrigeration temperature (4°C) until submission, at which time they should be packed on ice and sent via an expeditious method. If culture is desired, it is important to be clear on the accompanying paperwork that *Clostridium difficile* is suspected because special culture media and incubation conditions are required. Standard anaerobic culture will not be successful.

Although more work needs to be performed on the role of *C. difficile* in equine colitis, a positive toxin assay in combination with appropriate clinical signs is considered by the authors to be diagnostic for *C. difficile*-associated disease. If selective culture is positive, the diagnosis is reinforced, but negative culture does not indicate a likely false-positive result because of the poor survival of the organism. Culture-positive, toxin-negative results typically indicate the presence of a nontoxigenic and therefore clinically irrelevant strain of *C. difficile*, but the authors have cultured toxigenic strains from foals with colitis in which no toxin was detected in the feces. The role of *C. difficile* in such cases is unclear.

**References and Footnotes**


*Clostridium difficile* medium, Becton Dickinson Microbiology Systems, 2464 South Sheridan Way, Mississauga, Ontario, Canada L5J 2M8.

\(^a\)Pro-Disc, Remel, 12076 Santa Fe Dr., Lenexa, KS 66215.

\(^b\)Clostridium difficile* Tox A/B Test, TechLab, 1861 Pratt Dr., Suite 1030, Blacksburg, VA 24060-6364.

\(^d\)BBL Port-A-Cul Tubes, Becton Dickinson Microbiology Systems, 2464 South Sheridan Way, Mississauga, Ontario, Canada L5J 2M8.