Research Note

Clostridium difficile from Healthy Food Animals: Optimized Isolation and Prevalence†

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ABSTRACT

Two isolation methods were compared for isolation of Clostridium difficile from food animal feces. The single alcohol shock method (SS) used selective enrichment in cycloserine-cefoxitin fructose broth supplemented with 0.1% sodium taurocholate, followed by alcohol shock and isolation on tryptic soy agar supplemented with 5% sheep blood, and cycloserine-cefoxitin fructose agar. The double alcohol shock method (DS) used alcohol shock prior to and after selective enrichment in cycloserine-cefoxitin fructose broth supplemented with 0.1% sodium taurocholate, followed by isolation on tryptic soy agar supplemented with 5% sheep blood and cycloserine-cefoxitin fructose agar. A total of 55 (15.9%, n = 345) swine fecal samples, 32 (2.4%, n = 1,325) dairy cattle fecal samples, and 188 (6.3%, n = 2,965) beef cattle fecal samples were positive for C. difficile by either method. However, the DS was significantly better than the SS for the recovery of C. difficile from swine feces, while the SS was significantly better than the DS for the recovery of C. difficile from beef cattle feces. There was no significant difference between methods for the recovery of C. difficile from dairy cattle feces. This study suggests that food animals might harbor C. difficile and it provides critical information that isolation methods might not have universal application across animal species.

Clostridium difficile is an anaerobic, gram-positive, spore-forming bacillus that is commonly associated with a spectrum of diseases referred to as C. difficile-associated disease, which can range from uncomplicated mild diarrhea (4, 11) to life-threatening toxic megacolon and pseudomembranous colitis in humans (10). The infection is generally believed to be acquired nosocomially (25). However, community-acquired C. difficile–associated disease is increasingly reported (15, 20).

Few studies have been directed at elucidating the role of C. difficile as a potential cause of serious illness outside hospital environments (18, 19, 26). C. difficile has been isolated from domestic pets (1, 6, 16), food animals (12, 19, 22, 26), and retail meat (23, 28). Molecular typing of C. difficile isolates from pigs (9) and calves (22) has shown similarities in PCR ribotypes with human isolates, including two PCR ribotypes from calves that were associated with outbreaks of severe disease in humans in Canada (22), North America, and Europe (27). It is not clear whether these animals were the source of human C. difficile–associated disease. However, recent detection of C. difficile in retail meat markets along with a high degree of molecular similarity warrants further investigation (23, 28).

Several selective media and isolation procedures have been developed during the past three decades. Most of these procedures rely on an enrichment step to enhance the recovery of spores (2, 7, 17, 21). Alcohol shock has been added to effectively reduce the number of C. difficile vegetative cells and other interfering organisms (8, 14).

The objectives of this study were to compare the single alcohol shock method (SS), which utilizes selective enrichment in cycloserine-cefoxitin fructose broth supplemented with 0.1% sodium taurocholate (TCCFB), followed by alcohol shock and plating onto tryptic soy agar supplemented with 5% sheep blood (BA) and cycloserine-cefoxitin fructose agar (CCFA), with a double alcohol shock method (DS), which utilizes alcohol shock prior to and after selective enrichment in TCCFB and plating onto BA and CCFA, for the isolation of C. difficile from feces of swine, dairy cattle, and beef cattle. From these methods, we determined an apparent prevalence of C. difficile in healthy food animals that were sampled.

MATERIALS AND METHODS

Samples. From November 2006 to September 2008, a total of 4,635 fecal samples were collected from 345 grower–finisher...
swine, 1,325 adult dairy cattle, and 2,965 adult beef cattle (Table 1). All fecal samples were obtained from 35 states in the United States, as defined by the National Animal Health Monitoring System program (http://nahms.aphis.usda.gov). All animals were presumed to be healthy, without clinical signs of disease including diarrhea. The samples were shipped to the laboratory overnight on ice packs and processed on receipt.

### Culture methods

Two isolation methods (SS and DS) and two plating media (BA and CCFA; Remel, Lenexa, KS) were used for the isolation of *C. difficile*. All plating media and broth were prerduced in an anaerobic chamber (5% hydrogen, 5% CO₂, balanced nitrogen; Bactron Anaerobic, model BacII, Sheldon Manufacturing, Cornelius, OR) 24 h prior to use.

The SS method was performed as described by Arroyo et al. (2). The DS method was performed as follows. In brief, 2 g of fecal sample was mixed with 6 ml of absolute ethanol in a 15-ml conical tube and left at room temperature for 60 min. The sample was then centrifuged at 3,800 × g for 10 min at 4°C. The resulting pellet was disrupted with a swab, which was then used to inoculate 9.0 ml of prereduced TCCFB in screw-cap tubes and incubated aerobically at 37°C for 7 days. After incubation, 3.0 ml was transferred into a 15-ml conical tube, mixed with an equal amount of absolute ethanol, and left at room temperature for 60 min, at which time the sample was centrifuged at 4,600 × g for 30 min at 4°C. The supernatant fluid was discarded; the pellet was mixed with a sterile swab, which was subsequently used to streak prereduced BA and CCFA plates and incubated anaerobically in the aforementioned chamber at 37°C for 72 h (5% hydrogen, 5% CO₂, balanced nitrogen).

**Confirmation of *C. difficile***

Plates were examined for typical *C. difficile* colonies by using the following criteria: observation of yellow-green fluorescence UV light (350 nm) and production of a horse manure–like odor. Suspect colonies were subcultured to CCFA and incubated anaerobically at 37°C for 72 h for purity prior to further testing. After incubation, the colonies were observed for a flat, ground glass–like surface with irregular edge morphology, as well as the fluorescence and odor as described above. Additionally, a Gram stain was done to confirm that they were gram-positive and posed long, thin, straight rods under a ×1,000 light microscope. Biochemical confirmation included testing for the production of l-proline aminopeptidase (Pro-Disc, Remel, Carr-Scarborough Microbiologicals, Inc., Decatur, GA). Definitive confirmation was made with 16S rDNA PCR, as described by Kikuchi et al. (13). All positive isolates were stored in 10 ml of cooked meat medium in paraffilmed screw-cap tubes at room temperature after 48 h of anaerobic growth initiation at 37°C.

### Statistical analysis

A multivariable logistic regression model was constructed to assess the impact of media, isolation methods, and animal species on the test result. The model was implemented in SUDAAN 10.0 (Research Triangle International, Research Triangle Park, NC), which uses a Taylor series approximation to account for the lack of independence among samples taken from the same farm. All three independent variables (media, isolation methods, and animal species) were kept in the model, since the objective of the analysis was to assess the impact of the independent variable on the outcomes. Linear contrasts were constructed to investigate the interaction between isolation methods and animal species. All P values were two sided. All statements of statistical significance were based on P < 0.05.

## RESULTS

Recovery of *C. difficile* spores from the feces of food animals is shown in Table 2. Of the 345 swine fecal samples analyzed, 55 (15.9%) were positive for *C. difficile* by at least one of the two alcohol shock methods, regardless of culture media. Of the 55 positive samples, 22 and 50 were detected by the SS and DS, respectively. Feces from 32 (2.4%) of 1,325 dairy cattle were positive for *C. difficile* by at least one of the two alcohol shock methods, regardless of culture media. Of the 32 positive samples, 22 and 14 were detected by the SS and DS, respectively. Of the 2,965 beef cattle fecal samples analyzed, 188 (6.3%) were shown to have *C. difficile* present by at least one of the two alcohol shock methods, regardless of culture media. Of these 188 positive samples, 161 and 48 were detected by the SS and DS, respectively. Prevalence of *C. difficile* per farm ranged from 0 to 66, 0 to 33, and 0 to 100% in swine, dairy cattle, and beef cattle, respectively.

The media used did not affect the percentage of *C. difficile* isolation, regardless of isolation methods or animal species.

### TABLE 2. Percent total recovery of Clostridium difficile from the feces of food animals and percent by isolation method

<table>
<thead>
<tr>
<th>Isolation method</th>
<th>Swine % positive (no. positive/total no.)</th>
<th>Dairy cattle % positive (no. positive/total no.)</th>
<th>Beef cattle % positive (no. positive/total no.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Single alcohol shock</td>
<td>15.9 (55/345)</td>
<td>2.4 (32/1,325)</td>
<td>6.3 (188/2,965)</td>
</tr>
<tr>
<td>Double alcohol shock</td>
<td>40.0 (22/55)</td>
<td>68.8 (22/32)</td>
<td>85.6 (161/188)</td>
</tr>
<tr>
<td>Total recovered</td>
<td>90.9 (50/55)</td>
<td>43.8 (14/32)</td>
<td>25.5 (48/188)</td>
</tr>
</tbody>
</table>
TABLE 3. Percent recovery of Clostridium difficile from the feces of food animals by isolation methods and media

<table>
<thead>
<tr>
<th>Isolation method</th>
<th>Media</th>
<th>Swine (n = 345)</th>
<th>Dairy cattle (n = 1,325)</th>
<th>Beef cattle (n = 2,965)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Single alcohol shock</td>
<td>BA</td>
<td>5.8 (20)</td>
<td>1.1 (15)</td>
<td>4.9 (145)</td>
</tr>
<tr>
<td></td>
<td>CCFA</td>
<td>4.9 (17)</td>
<td>1.4 (19)</td>
<td>5.3 (157)</td>
</tr>
<tr>
<td></td>
<td>BA/CCFA</td>
<td>6.4 (22)</td>
<td>1.7 (22)</td>
<td>5.4 (161)</td>
</tr>
<tr>
<td>Double alcohol shock</td>
<td>BA</td>
<td>13.0 (45)</td>
<td>0.8 (10)</td>
<td>1.3 (40)</td>
</tr>
<tr>
<td></td>
<td>CCFA</td>
<td>12.8 (44)</td>
<td>1.0 (14)</td>
<td>1.0 (31)</td>
</tr>
<tr>
<td></td>
<td>BA/CCFA</td>
<td>14.5 (50)</td>
<td>1.0 (14)</td>
<td>1.6 (48)</td>
</tr>
</tbody>
</table>

* BA, tryptic soy agar supplemented with 5% sheep blood; CCFA, cycloserine-cefoxitin fructose agar; BA/CCFA, either of both methods.

species. However, there was a significant association between isolation method and animal species (P < 0.001). The percent recovery of C. difficile by isolation method and media is shown in Table 3. In swine feces, the DS detected significantly more C. difficile–positive samples than did the SS, regardless of media used (odds ratio [OR] = 2.63, P = 0.00018). Conversely, in beef cattle feces, the SS detected significantly more C. difficile–positive samples than did the DS, regardless of media used (OR = 4.43, P < 0.0001). For dairy cattle feces, there was no significant difference of C. difficile isolation between any combination of isolation methods and media. Across animal species, the SS detected a similar percent positive for swine and beef feces, but the DS for swine feces detected a much higher percent positive than it did for beef feces (OR = 12.22, P < 0.0001).

DISCUSSION

Results indicate that swine are more likely to be positive for C. difficile than are dairy or beef cattle. Our study is consistent with recent Slovenian reports (3, 19), although their reported isolation rates in swine were higher than our rates were. This could be attributed to the fact that they sampled piglets, whereas we sampled much older animals. Conversely, Russell (24) reported that Clostridium spp. were observed at a prevalence rate of 5.3% in swine. The lower observed prevalence rates of C. difficile from dairy and beef cattle feces might be attributed to several factors including differences in diet, digestive system architecture, and microbial diversity of the gastrointestinal tract compared with swine.

Comparison of alcohol shock for the recovery of C. difficile also demonstrated difference in recovery by isolation method and species. The difference in recovery between species is also interesting and while it is significant, it might be attributed to diet and other environmental factors in addition to the tests themselves. One explanation may be that a second alcohol shock might further damage injured C. difficile spores, resulting in a lower survival rate as compared with strains among swine samples. Additionally, cattle and swine have different digestive systems and diets; cattle are ruminants. However, the low prevalence suggests that C. difficile is less common in cattle. In addition, the concentration of C. difficile spores in the intestinal tract might not represent the same concentration in fecal samples.

Microorganisms other than C. difficile frequently grew on BA in large numbers, making isolation of C. difficile in pure culture difficult because of the necessity for repeated subculture. On the other hand, C. difficile could readily be distinguished from the other organisms that occasionally grew on CCFA. CCFA is the medium of choice to isolate C. difficile from feces when compared with BA.

Our efforts have been directed toward improving techniques for the recovery of C. difficile spores from food animal feces. These data demonstrate that recovery of C. difficile spores is less dependent on medium and more dependent on use of alcohol shock. The presence of C. difficile spores in the feces of swine, dairy cattle, and beef cattle supports a potential risk for contamination of pork and beef products during slaughter and milk during milking, although these risks cannot be quantified from these data. While proper cooking and milk pasteurization are emphasized for reducing the risk of bacterial illness, the fact that C. difficile is a spore former complicates this issue. Characterization, molecular typing, and epidemiological studies are needed to establish the relationship between food animal isolates and those from humans to determine the true potential for acquisition of foodborne disease.

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REFERENCES


