Hyperimmune Bovine Colostrum as a Novel Therapy to Combat Clostridium difficile Infection

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Background. Clostridium difficile is a primary cause of antibiotic-associated diarrhea that typically develops when gut microbiota is altered. Conventional treatment for C. difficile infection (CDI) is additional antimicrobial administration, which further disrupts normal intestinal microbiota, often resulting in poor treatment outcomes.

Methods. A pregnant dairy cow was repeatedly immunized with recombinant mutants of toxins A and B produced by C. difficile, and the resultant hyperimmune bovine colostrum (HBC) was evaluated for therapeutic efficacy in gnotobiotic piglets with diarrhea due to CDI. Control piglets received nonimmune colostrum. To determine the impact of HBC on gut microbiota, 1 of 2 groups of piglets transplanted with normal human gut microbiota was treated with HBC.

Results. Nonimmune colostrum–treated piglets developed moderate to severe diarrhea and colitis. In contrast, HBC-treated piglets had mild or no diarrhea and mild or no colitis. Lyophilization had no detectable impact on HBC efficacy. HBC had no discernible effect on the composition of normal human gut microbiota in the porcine intestinal tract.

Conclusions. HBC provides an oral, cost-effective, and safe alternative to antibiotic therapy for CDI. By preserving intestinal microbiota, HBC may be more efficacious than antibiotics. Additional studies are warranted to establish HBC as a viable immunotherapeutic agent for human use against CDI.

Keywords. Clostridium difficile; hyperimmune bovine colostrum; intestinal microflora; intestinal microbiota; immunotherapeutic.
pathogens. Frequent, repeated inoculation of a gestating dairy cow may stimulate increased production of high levels of colos- 

tral immunoglobulin against a targeted antigen, resulting in hy- 

perimmune bovine colostrum (HBC). HBC has previously been 
generated and shown efficacy as a treatment or preventive 
against enteric pathogens including C. difficile, Cryptosporidi- 
um, Escherichia coli, rotavirus, and Shigella [16].

Pathology and clinical signs associated with CDI have been 
linked to the presence of toxins A and B produced by C. difficile 
(TcdA and TcdB) [17]. Previous work has shown that C. difficile 
toxin–specific HBC neutralized cytotoxicity of TcdA and TcdB 
in human fibroblasts [18], and colostrum inhibited adhesion of 
C. difficile to human enterocytes [19]. CDI was prevented in 
hamsters given HBC before C. difficile challenge [20]. In rats, 
HBC inhibited enterotoxic effects of TcdA and TcdB [18]. In 
humans, C. difficile toxin–specific antibodies in HBC survived 
passage through the gastrointestinal tract and were subsequently 
able to neutralize TcdA and TcdB [21, 22]. HBC has proved at 
least as effective as metronidazole in treating recurrent CDI [7], 
and it has also shown promise in preventing relapse [23].

Here we describe the immunization of a pregnant cow with 
highly purified and concentrated recombinant TcdA and TcdB 
mutants, which resulted in the production of 3 gallons of HBC 
rich in specific colostral immunoglobulins against the 2 toxins. 
This HBC, when fed in liquid or powder form, led to a rapid 
recovery of piglets with acute diarrhea caused by CDI. HBC 
treatment had no effect on the integrity of the gut microbiota 
of human origin.

MATERIALS AND METHODS

Generation of HBC
A pregnant Holstein cow from Jordan Dairy, Rutland, Massa- 
chusetts, was hyperimmunized using 200 µg each of atoxic re- 
combinant TcdA and TcdB, which were prepared in our 
laboratory as described elsewhere [24]. Beginning at 32 weeks 
of gestation, the cow received subcutaneous inoculations using 
aluim as an adjuvant, every 2 weeks for a total of 4 injections. 
An intramammary infusion of 400 µg each of atoxic recombinant 
TcdA and TcdB, using modified labile toxin of enterotoxigenic 
E. coli as an adjuvant [25], was divided evenly among the 4 quar- 
ters at the time of the final subcutaneous injection. Samples taken 
at each immunization were used to assess rising levels of serum 
immunoglobulin G against TcdA and TcdB.

During the first 12 hours after parturition, HBC was harvested 
by hand milking, separated into 25-mL aliquots, and frozen at 
~20°C. Some HBC was lyophilized using a lyophilizer (Freeze- 
mobile 25XL; Virtis). For control, nonimmune colostrum was 
obtained from another Jordan Dairy cow of the same parity 
that gave birth and began lactating at the same time. Colostrum 
samples were cultured on 5% sheep’s blood agar and incubated 
aerobically for 48 hours to assess bacterial contamination.

Animals
Twenty-three gnotobiotic piglets from 4 litters were born by 
cesarean delivery, placed in sterile isolators, and fed Similac 
(Abbott) milk replacer 3 times daily [26]. Nineteen pigs were 
orally inoculated with 10^7 C. difficile spores at 5 days of age. 
At 6 days of age, 5 pigs were orally treated with 25 mL of frozen 
thawed HBC, 5 were treated with an equivalent volume of ly- 
ophilized HBC (reconstituted with milk replacer), and 9 were 
treated with 25 mL of frozen thawed nonimmune bovine colos- 
trum, twice daily for 7 days. Daily fecal samples were collected 
from all 19 piglets for the duration of the experiment, beginning 
before inoculation.

The piglets were closely observed several times daily for clin- 
cical signs of CDI, including diarrhea, dehydration, lethargy, an-
orexia, and weakness. They were euthanized at a predetermined 
end point after treatment with colostrum for 7 days, or sooner if 
they exhibited severe signs of illness, such as anorexia or weak-
ness. Blood was collected from all piglets before inoculation 
with C. difficile and again before euthanasia. Cecal, spiral 
colon, and rectal contents were collected at necropsy. Kidney, 

duers, spleen, and large intestinal tissue samples including 
cecum, spiral colon, and rectum were collected and fixed in for- 
malin for histopathologic examination.

Four additional gnotobiotic pigs were each given 3 mL of 
normal human gut microbiota suspension by oral gavage at 5 
days of age. Beginning at 7 days of age, 2 pigs were fed 25 mL 
of HBC twice daily for 7 days. Fecal samples were obtained from 
each pig every other day until the experimental end point, be-
ginning before administration of normal human gut microbio-
ta. All 4 pigs were euthanized at 14 days of age. Gross necropsies 
were performed, and organ samples were fixed in formalin. His-

topathologic examination of all tissues was performed by a 
board-certified veterinary pathologist (G. B.). This study was 
approved by Tufts University Institutional Animal Care and Use 
Committee.

Preparation of C. difficile Inoculum
The inoculum was prepared with C. difficile strain UK6, type 
027/B121/NAP1, producing TcdA and TcdB. Colonies grown 
on brain-heart-infusion agar were incubated anaerobically in 
brain-heart-infusion broth at 37°C for 10 days. After centrifuga-
tion and washing, the suspension was heated to kill vegetative 
cells, and the remaining spores were stored at 4°C [26]. The 
concentration was adjusted to contain 10^7 spores per 2 mL 
per inoculated piglet.

Bacterial Culture and Counts
Fecal samples collected before C. difficile inoculation were cul-
tured for contaminants by streaking on 5% sheep’s blood and 
incubating aerobically at 37°C for 48 hours. Daily fecal samples 
and large intestinal contents obtained at necropsy were plated 
on C. difficile–selective taurocholate-cefoxitin-cycloserine-fructose
agar in serial 10-fold dilutions and incubated anaerobically at 37°C for 48 hours to determine onset of bacterial shedding and bacterial counts.

**Cytotoxicity Assay**

The presence of TcdA and TcdB was evaluated in large-intestinal samples collected at necropsy [27]. CT26 murine colonic carcinoma cells were incubated overnight at 37°C in a 96-well plate with Dulbecco’s modified Eagle medium, 10% fetal bovine serum, 1% L-glutamine, 1% sodium pyruvate, and 0.5% penicillin/streptomycin. Fecal samples were passed through a 0.45-µm syringe filter and then added to the cell culture in serial dilutions (100 µL/well). Recombinant TcdA and TcdB (both 10 ng/mL) were added as positive controls, and the plate was incubated for 24 hours at 37°C before visual assessment of cell rounding.

**Table 1. Summary of Findings in Piglets With CDI Treated With HBC or Nonimmune Colostrum**

<table>
<thead>
<tr>
<th>Clinical signs</th>
<th>HBC-Treated Pigs (n = 10)</th>
<th>Nonimmune Colostrum–Treated Pigs (n = 9)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mild to resolved diarrhea (n = 10), good appetite (n = 10)</td>
<td>Moderate to severe diarrhea (n = 7), anorexia, weakness (n = 4), anal swelling (n = 3)</td>
<td></td>
</tr>
<tr>
<td>Gross lesions</td>
<td>None (n = 10)</td>
<td>Moderate mesocolonic edema and congestion of spiral colon (n = 9)</td>
</tr>
<tr>
<td>Histopathologic lesions</td>
<td>None (n = 3), mild large-intestinal inflammation (n = 7)</td>
<td>Moderate to severe neutrophilic colitis (n = 9), multifocal epithelial ulceration (n = 7), diphtheritic cecal membrane (n = 2)</td>
</tr>
<tr>
<td>Large-intestinal C. difficile count, mean (SD), CFUs/mL^a</td>
<td>8.3 × 10^10 (1.8 × 10^11) (n = 10)</td>
<td>1.4 × 10^9 (3.2 × 10^9) (n = 8)</td>
</tr>
<tr>
<td>Presence of TcdA or TcdB in fecal samples</td>
<td>Positive (n = 10)</td>
<td>Positive (n = 9)</td>
</tr>
<tr>
<td>Fecal IL-1-β^a</td>
<td>21 041 (29 122) (n = 5)</td>
<td>18 912 (7875) (n = 4)</td>
</tr>
<tr>
<td>Fecal IL-8^a</td>
<td>1060 (1218) (n = 5)</td>
<td>2291 (1071) (n = 4)</td>
</tr>
<tr>
<td>Serum IL-1-β^a</td>
<td>139 (278) (n = 5)</td>
<td>186 (321) (n = 4)</td>
</tr>
<tr>
<td>Serum IL-8^a</td>
<td>140 (31) (n = 5)</td>
<td>271 (268) (n = 4)</td>
</tr>
</tbody>
</table>

Abbreviations: C. difficile, Clostridium difficile; CDI, C. difficile infection; CFUs, colony-forming units; HBC, hyperimmune bovine Colostrum; IL-1-β, interleukin 1-β; IL-8, interleukin 8; SD, standard deviation; TcdA, toxin A produced by C. difficile; TcdB, toxin B produced by C. difficile.

^a P > .05 (Mann–Whitney U test) for difference between treatment groups.
Cytokine Measurement

Cytokine levels of interleukin 1-β and interleukin 8 in both fecal and serum samples were measured before inoculation and at necropsy on the same experimental day using commercially available porcine cytokine enzyme-linked immunosorbent assay kits (Quantikine ELISA; R & D Systems). Samples were diluted 1:10 with sterile phosphate-buffered saline, thoroughly mixed using a vortex, and centrifuged, and the supernatant was added to reagent wells in the assay. The assay was performed according to the manufacturer’s instructions, and cytokine concentrations were determined based on the standard curve.

Preparation of Normal Human Gut Microbiota Suspension

Normal human gut microbiota suspension was prepared by pooling fecal samples from 10 healthy adults (5 women and 5 men), aged 50–70 years. Equal amounts of each human fecal sample were diluted 1:10 (wt/vol) in prereduced sterile phosphate-buffered saline in an anaerobic chamber. The stock material was amended with sterile glycerol to a final concentration of 10% and stored at −80°C [28].

Microbiome Analysis

Total DNA representing human gut microbial communities was extracted from individual fecal samples as previously described [29]. Briefly, samples were diluted and alternately frozen (−80°C for 6–9 minutes) and thawed (37°C, 2–4 minutes) for a total of 5 cycles. Extraction was performed using the High Pure PCR Template Preparation Kit (Roche) with universal primers flanking the V1–V2 hypervariable regions of the bacterial 16S ribosomal RNA genes. Polymerase chain reaction products were tagged with a 6-nucleotide barcode unique to each sample and pooled to generate a library that was sequenced in a
HiSeq2000 Illumina sequencer at Tufts University Core Facility (tucf.org). Sequence reads with ambiguous base calls, sequences with <300 bases, and chimeras were eliminated [30]. Phylogenetic assessments were performed using the Ribosomal Database Project classifier implemented in QIIME software (an open source software powered by Pycogent) with a bootstrap cutoff of 80%, and a principal coordinates analysis plot was generated with the unweighted distance metric [31, 32].

RESULTS

Immunization with Recombinant Toxins and Production of Antitoxin Antibodies

Rising titers to TcdA and TcdB were measured in bovine serum samples collected at baseline and subsequent immunizations (Figure 1A). Anti-TcdA and anti-TcdB antibodies were also assessed in both liquid and lyophilized postparturient colostrum, and antibody titers were highest in liquid HBC, followed by lyophilized HBC (Figure 1B). HBC successfully neutralized TcdA and TcdB in vitro.

Effect of HBC in Preventing Clinical Signs and Lesions of CDI

All 19 piglets developed mild diarrhea before initiation of colostrum treatment. All 10 pigs fed HBC had mild or resolved diarrhea and good appetites at the experimental end point. There was no difference between pigs treated with liquid and those treated with lyophilized HBC. Of 9 pigs fed nonimmune colostrum as a control, 7 developed moderate to severe watery diarrhea, 3 had anal swelling, and 4 were euthanized before the experimental end point owing to onset of severe clinical signs of CDI, including anorexia and weakness (Table 1). Pigs treated with HBC had no gross lesions. All 9 treated with nonimmune colostrum had moderate mesocolonic edema and congestion of the spiral colon (Figure 2). All 10 pigs fed HBC showed mild to no large-intestinal inflammation and no epithelial ulceration histologically.

Analysis of large-intestinal sections collected at necropsy revealed moderate to severe neutrophilic colitis in all 9 pigs fed nonimmune colostrum, whereas 7 of 9 had multifocal epithelial ulceration, and 2 pigs showed evidence of a pseudomembrane.
lining the cecum. A quantitative assessment of colitis severity was performed by counting neutrophilic foci in spiral colon sections from each pig. Foci were observed between colonic crypts in the lamina propria in 10 random fields with ×20 magnification. Pigs treated with HBC had significantly fewer foci of neutrophils within histopathologic sections of spiral colon than those treated with nonimmune colostrum (P < .001; Mann–Whitney U test).

Among the 4 pigs populated with normal human gut microbiota, there was no clinical difference between the 2 pigs treated with HBC and the controls. None showed signs of illness, and all ate well for the duration of the experiment. All piglets treated with normal human gut microbiota were lacking gross lesions at necropsy, and no significant microscopic lesions were discerned at histopathology.

**Laboratory Results Produced by HBC and Nonimmune Colostrum**

Fewer than 20 colony-forming units per plate were observed after bovine colostrum was streaked on sheep’s blood agar. Porcine fecal samples cultured before inoculation produced no growth, indicating absence of contaminants. Daily fecal samples demonstrated *C. difficile* shedding in all pigs, beginning within 48 hours after inoculation. There was no significant difference between *C. difficile* counts from large-intestinal contents from HBC-treated pigs and pigs treated with nonimmune colostrum (Table 1). Fecal samples from all pigs resulted in cell rounding at 24 hours, probably owing to the presence of TcdA and TcdB. Interleukin 1-β and interleukin 8 were detected in fecal and serum samples of HBC and nonimmune colostrum–treated pigs, but there were no significant differences in mean cytokine levels in fecal or serum samples between groups.

**Effect of HBC Treatment on Gut Microbiota**

Bacterial communities from 4 piglets given normal human gut microbiota consisted primarily of Bacteroidetes, Firmicutes, and Proteobacteria. There was no detectable difference in gut bacterial composition between HBC-treated piglets and controls (Figure 3). The principal coordinates analysis plot shows similar compositions of microbiota in samples taken from pigs with and without HBC treatment at different, matched time points (Figure 4).

**DISCUSSION**

This work demonstrates that *C. difficile* toxin–specific HBC is effective against CDI and does not seem to disrupt normal human gut microbiota. Bovine colostrum is currently available to the public in oral, over-the-counter preparations, and it is a popular daily dietary supplement alleged to enhance athletic performance and immune function [33, 34]. In human clinical trials, bovine colostrum has shown promise as an effective therapy for colitis [35], cryptosporidiosis [36], failure to thrive [37], human immunodeficiency virus–associated diarrhea [38], recurrent CDI [7], and rotavirus [39, 40]. Bovine colostrum has also demonstrated potential as a preventive for drug-associated gastroenteropathy [41], enterotoxigenic *E. coli* diarrhea [42], and shigellosis [43]. In all cases, colostrum has been well tolerated, with no untoward adverse effects reported. Given the available infrastructure for milk production and its relatively low expense, HBC could be generated in bulk as a safe, cost-effective therapy against CDI, without threatening commensal microbial inhabitants of the intestinal tract.

HBC has been administered in several formulations, ranging from whole HBC to immunoglobulin concentrate to purified immunoglobulin [16]. Previous work has demonstrated the efficacy of liquid, whole HBC as a treatment for cryptosporidiosis [44, 45] and rotavirus [39]. Dried, whole HBC has been employed as a preventive against *E. coli* infection [42]. Anti–*C. difficile* immunoglobulins have been purified from HBC and used to successfully treat CDI [7] and relapse [23]. To our knowledge, this is the first time a whole-HBC product (in both liquid and lyophilized forms) has been used to effectively treat CDI in the piglet diarrhea model. Like humans, pigs develop severe gastrointestinal lesions due to *C. difficile* toxins, including pseudo-membranous colitis and systemic disease [26].

Our work concurs with previous findings demonstrating that immunoglobulin integrity is maintained during lyophilization [46]. Liquid and lyophilized HBC were equally effective against CDI. Whole HBC may confer an added therapeutic benefit over an immunoglobulin–only product, because even nonimmune
colostrum, without specific antibodies, is known to possess innate factors, including lactoferrin, cytokines, and growth factors, that may contribute synergistically to the role of colostrum as an agent of passive immunity [47,48]. Indeed, several control pigs receiving nonimmune colostrum appeared to derive some benefit from their treatment, because they did not all develop fulminant clinical disease nor the severe histopathologic lesions previously observed in the gnotobiotic piglet model of CDI [26]. Further studies of the antibody fraction of HBC may be indicated to more fully characterize the effects of anti-TcdA and anti-TcdB alone.

This study initiated HBC treatment 24 hours after C. difficile inoculation, and although all pigs developed diarrhea before therapy started, none was otherwise showing clinical signs of illness. Given that CDI in humans typically causes significant symptoms and disease well before patients receive treatment, future studies allowing more time for C. difficile to establish infection before therapy are warranted. Finally, HBC dosage, frequency of administration, and duration of treatment necessary to achieve clinical resolution should be further explored.

As the population ages, more persons will probably undergo antibiotic treatment, spend additional time in hospitals, and unwittingly bolster the presence of CDI in the healthcare landscape. Conventional treatment of CDI employing extended and repeated courses of antimicrobial therapy increases the likelihood of enhanced antibiotic resistance. Clostridium difficile toxin–specific HBC presents a novel immunoglobulin-driven treatment to control CDI, while sparing colonic microbiota. In addition to providing an efficacious first-line treatment for CDI, HBC therapy may (given the lack of appreciable impact on gut microbiota) also reduce the risk of disease recurrence, the most common sequela of CDI.

HBC has the potential to curtail the financial burden of treating CDI. A single, uncomplicated case may cost nearly $5000 to treat, and expenses incurred by recurrent CDI may exceed $18 000 for a patient [49]. A recent analysis found that C. difficile–related medical expenditures in American hospitals total almost 5 billion dollars annually, and the economic impact of CDI on long-term care facilities is yet undetermined [50]. The cost of generating HBC would be small in comparison. HBC should be considered for further evaluation as a promising immunotherapeutic agent for CDI. Successful development of HBC as an effective, oral, safe, affordable alternative to antibiotic treatment could improve patient outcomes, trim healthcare costs, and diminish the mounting threat to public health that CDI now poses.

Notes

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