Fecal Microbiota Transplant for Relapsing *Clostridium difficile* Infection Using a Frozen Inoculum from Unrelated Donors – a Randomized, Open Label, Controlled Pilot Study.

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Keywords: Fecal Microbiota Transplant; Clostridium difficile; microbiome; frozen inoculum.

Short title: Frozen, unrelated FMT for *C. difficile*

Summary: Fecal microbiota transplant is increasingly used to treat recurrent or relapsing *C. difficile*. In this randomized, controlled study; using a frozen inoculum from unrelated donors was safe and effective; whether administered by nasogastric tube or by colonoscopy.

Abstract:

Background: Recurrent *C. difficile* infection (CDI) is a growing medical concern with poor response to standard antimicrobial therapy. We aimed to investigate the outcomes of fecal microbiota transplant (FMT) for relapsing CDI using a frozen suspension from unrelated donors, comparing colonoscopic and nasogastric-tube (NGT) administration.

Methods: Healthy volunteer donors were screened and a frozen fecal suspension was generated. Patients with relapsing/refractory CDI were randomized to receive an infusion of donor stools by colonoscopy or NGT. The primary endpoint was clinical resolution of diarrhea without relapse after 8 weeks. Secondary endpoints were self-reported health score using standardized questionnaires.

Results: A total of 20 patients were enrolled, 10 in each treatment arm. Patients had a median (range) of 4 (2-16) relapses prior to study enrollment, with 5 (3-15) antibiotic treatment failures. Resolution of diarrhea was achieved in 14 (70%) after a single FMT (8 of 10 in the colonoscopy group and 6 of 10 in the NGT group). Five patients were retreated with 4 obtaining cure, resulting in an overall cure rate of 90%. Daily number of bowel movements changed from a median (IQR) of 7 (5-10) the day prior to FMT to 2 (1-2) after the infusion. Self-ranked health scores improved significantly from a median (IQR) of 4 (2-6) pre-transplant to 8 (5-9) post-transplant. No serious or unexpected adverse events occurred.

Conclusion: In our initial feasibility study FMT using a frozen inoculum from unrelated donors is effective in treating relapsing CDI. NGT administration appears to be as effective as colonoscopic administration.

Background:

Recurrent and refractory *Clostridium difficile* infection (CDI) is a growing medical concern with a recent dramatic increase in the number of patients globally [1–4]. In the United States the incidence of CDI has tripled over the last 15 years [3]. Response to standard antimicrobial therapy with oral vancomycin or metronidazole is suboptimal, with CDI recurring in up to 30% of individuals treated for a first episode. After two or more episodes of CDI the estimated risk for subsequent recurrence exceeds 60% with antimicrobial therapy [3,5–8]. Often patients with recurrent CDI are treated with prolonged administration of oral vancomycin with tapering of the medication over many months, but this approach is poorly studied. The emergence of a virulent strain of the organism (NAP1/BI/027) has been associated with even higher rates of treatment failure [9,10]. The consequences of recurrence can be devastating, resulting in life-threatening illness, frequent hospitalizations and possible surgical interventions. In addition to individual morbidity and mortality, CDI taxes the medical system by requiring patient cohorting, leading to bed closures, delay of discharge, and additional contact precautions.

Although the illness is toxin-mediated, overgrowth of the organism in the setting of dysbiosis is thought to be a key inciting event. Failure to reconstitute normal flora was shown to be a factor in severe, recurrent, and prolonged illness [13]. Fecal microbiota transplantation (FMT) - reconstitution of normal flora by a stool transplant from a healthy individual - has been a successful therapeutic approach to recurrent/refractory CDI in animal studies [14], numerous case series [15–20] and, more recently, a single randomized clinical trial [21]. Even though an overall CDI resolution rate of about 90% has repeatedly been reported in published reviews and meta-analyses [22–25], practical and aesthetic barriers have hindered the widespread use of

FMT to date. Recruitment and screening of donors is a lengthy process associated with significant costs, thus preventing the use of FMT in acute situations. Establishing a repository of prescreened frozen donor stools could make this treatment available for a wider population. Furthermore, many questions remain regarding the optimal protocol donor screening, sample processing, route of administration and amount of fecal material instilled.

In the current study we aimed to investigate the clinical outcomes of FMT for refractory or relapsing CDI using a frozen suspension from unrelated donors by both upper and lower gastrointestinal routes.

Methods:

This was an open-label, randomized, controlled trial evaluating the efficacy of FMT in treating relapsing or recurrent CDI in a pilot cohort of 20 patients, comparing colonoscopic and nasogastric-tube (NGT) administration. The study was approved by the Partners Human Research Committee as well as by the United States Food and Drug Administration (IND # 15199) and registered at Clinical Trials.gov (NCT01704937). Candidates were recruited by referrals from colleagues at Partners' Healthcare of which MGH is a founding member. All adult participants provided written informed consent after a clinical meeting with a physician investigator providing information about potential risks and benefits of the procedure. Children aged 7 and above provided assent, in addition to parental informed consent. Participants were allocated to treatment arms by computer-generated randomization in blocks of four.

Study Population and Settings: The study was conducted at Massachusetts General Hospital. Included were subjects ages 7-90 with refractory or recurrent CDI, as defined in consensus

guidelines [26] by a relapse of CDI after having at least three episodes of mild-to-moderate CDI and failure of a 6-8 week taper with vancomycin with or without an alternative antibiotic, OR, at least two episodes of severe CDI resulting in hospitalization and associated with significant morbidity. Active CDI was defined as diarrhea (>3 loose stools per day) with a positive stool test for *C.difficile* toxin. Our hospital laboratory performs an initial toxin/glutamate dehydrogenase (GDH) ELISA, followed by PCR if only the GDH test is positive or indeterminate and does not routinely test for the NAP-1/B1/207 strain. Exclusion criteria included presence of anatomic contraindication to NGT or colonoscopy, delayed gastric emptying syndrome, recurrent aspirations, pregnancy, significantly compromised immunity (immunosuppressive medications, recent chemotherapy, decompensated liver cirrhosis, advanced HIV/AIDS [CD4 count<250], neutropenia with ANC <1000/ul, recent bone marrow transplant, or other cause of severe immunodeficiency) and having a history of significant allergy to foods not excluded from the donor diet. Stable oral prednisone treatment up to 40 mg daily was allowed.

Donor Screening: Donors were healthy, non-pregnant adults 18-50 years of age, on no medications, with a normal Body Mass Index (BMI 18.5-25). Volunteers were excluded for any significant past medical history (with the exception of resolved traumatic injury) or any use of antibiotics in the preceding 6 months. Candidates were initially screened using the American Association of Blood Banks donor questionnaire for exposure to infectious agents [27], then underwent physical examination and general laboratory screening tests (within 30 days of donations), including complete blood count with differential, renal function and electrolytes, complete liver function tests, albumin and total protein, lipid profile, high resolution C-reactive protein, fluorescent anti-nuclear antigen, and fecal occult blood testing. All results had to be

within normal range for age and gender. Donor feces were screened for enteric bacterial pathogens including rotavirus, *Listeria monocytogenes*, and *Vibrio cholerae*, *Escherichia coli* O157, ova and parasites (including general microscopy, acid-fast staining and/or antigen testing for giardia and cryptosporidium, isospora and microsporidia), *C. difficile* and *Helicobacter pylori* antigen. Blood was screened for antibodies to hepatitis A, B and C; HIV and *Treponema pallidum* within 2 weeks of donations. The volunteers were asked to refrain from eating common allergens within 5 days of stool donation (tree nuts, eggs, peanuts, shellfish) but otherwise not to alter their diets. At the time of donation they had an interim health query for febrile, systemic and GI symptoms and were deferred for any change in health status. Finally, all donations were escrowed for an additional four weeks, to allow re-testing of donors for HIV and hepatitis B and C prior to clinical use of the inoculum.

Preparation of frozen inocula: Donors were asked to take a dose of Milk of Magnesia the day before fecal collections in order to facilitate manipulation of the sample. A fecal suspension was generated in normal saline without preservatives, using a commercial blender. Materials were sequentially passed through four sieves to remove particulate material. The final slurry was concentrated 3-fold by centrifugation and then re-suspended in sterile saline with 10% glycerol added as a bacterial cryoprotectant. Inocula were then frozen at -80^{o} C pending use. The work of Hamilton and Khoruts [28] was used as a guide for fecal manipulation, with the exception that all processes were carried out under ambient air, not nitrogen. Each sieved inoculum was calculated at the conclusion of the project to have been derived from approximately 41 grams of fecal material. Inocula used in this study were stored frozen for up to 156 days (range 29-156). Frozen material was thawed in a 37^{0}c water bath, and then kept on ice until delivery.

Study procedures: (Supplementary Figure S1) Patients were required to discontinue all antibiotics at least 48 hours prior to the procedure. Subjects assigned to colonoscopic administration underwent a standard bowel preparation with four liters of Polyethylene glycol electrolyte solution, followed by endoscopic administration to the right colon of 90cc thawed inoculum. This amount of fecal material was further diluted to 250cc for adults and 160 cc for pediatric patients. Patients were asked to retain the material as long as possible post-procedure and were given a single oral dose of loperamide at the time of the procedure. Subjects assigned to NGT delivery of FMT were prescribed 2 mg/kg/day up to 20 mg of omeprazole orally for 48 hours prior to the procedure. An age and size-appropriate NGT was inserted, proper positioning in the stomach was documented by radiography and 90cc inoculum was administered. In these patients the inoculum was not further diluted, in order to minimize risk of vomiting and aspiration. The NGT was removed promptly after administration and subjects were asked to drink a glass of water to facilitate dilution of stomach contents and transit into the small intestine.

Patients in both study arms that showed no improvement in diarrheal symptoms were offered a second FMT by their preferred route of administration. In order to minimize potential infectious exposures, inoculum from the same donor was used for the repeat administration.

Patients in both groups were followed with structured questionnaires administered on days 1, 2, 3, 7, 14, 21 and at 2 and 6 months after the procedure (primarily by phone). Questionnaires recorded stool frequency and consistency, general well-being on a standardized health score, rating of gastrointestinal symptoms, medication use, weight changes, and elicited possible

adverse events by use of a modification of the Common Terminology Criteria for Adverse Events v3.0 [29] approved by the FDA and IRB.

Outcomes: The primary endpoint was clinical resolution of diarrhea off antibiotics for *C. difficile,* without relapse within 8 weeks. For patients that required a second treatment dose, follow up was calculated starting at the time of the second administration. Resolution of diarrhea was defined as fewer than three bowel movements per 24hrs**.** Secondary endpoints included improvement in subjective well-being per standardized questionnaire and presence of adverse events.

Data analysis: Continuous variables are presented as mean and standard deviation (SD), median and interquartile range (IQR). Categorical variables are presented as number and percentage of patients within each treatment group. Patient characteristics at baseline were compared between the two treatment groups in order to estimate the efficacy of randomization. The Mann-Whitney test was used for comparisons of continuous variables (patient characteristics and outcomes) between the two treatment groups and Fisher's exact test for comparisons of categorical variables. Outcomes were analyzed according to the intention-to-treat principle, with imputation of data by the last outcome carried forward. A mixed-model ANOVA was used to estimate difference in outcomes between the two treatment groups over the study time.

All statistical tests were two-sided; a p-value < 0.05 was considered statistically significant. Data were analyzed using SPSS statistical software version 21 (SPSS Inc., Chicago, IL, USA).

Analysis of fecal microbiota: A donor sample was collected at time of donation. Recipients provided stool samples before FMT, weekly for three weeks and then at 2 and 6 months. All fecal samples were stored at -80 C. DNA was extracted and the V4 region of the 16S gene was sequenced using an Illumina MiSeq (Illumina, San Diego, CA) as described previously [30]. The Shanon Diversity Index was computed for each sample and a custom python script was used to create summary plots illustrating the relationship between clinically relevant groupings and the diversity observed in the microbiome. We used the Shannon Diversity Index as our primary measure of diversity because it takes into account both abundance and evenness of species present in the community and has been shown to most robustly accommodate the variation in sampling depth [31]. See Supplementary Appendix 1 for detailed methods.

Results:

From December 2012 through May 2013, a total of 20 patients were randomly assigned to receive FMT via colonoscopy or NGT (Fig. 1). Baseline characteristics were comparable between groups (Table 1).

Donors: Of 37 candidates that responded to our call for volunteers, 12 passed the initial screening and underwent a full donor work-up. Seven were excluded from donating based on abnormal screening labs: 4 with positive anti-nuclear antibodies, 1 with elevated bilirubin, 1 with mild neutropenia and 1 with eosinophilia. The remaining 5 donors provided 3 stool samples each, that were used to generate 25 infusions used in 20 study patients.

Primary Outcome: Of 20 patients in both study arms, 14 were cured after the first infusion of donor feces (70%); 8 in the colonoscopy group (80%) and 6 in the NGT group (60%; p=0.628). One patient in the NGT arm refused subsequent re-treatment. The remaining 5 patients were

given a second infusion at mean 4.9 (SD 2.1) days after the first procedure, using feces from the same donor that provided the initial inoculum. Per protocol, patients could choose the route of delivery for re-treatment; and all 5 requested NGT administration. 4 patients obtained cure after the second inoculation, resulting in an overall cure rate of 90% (80% in the NGT group and 100% in the initial colonoscopy group, p=0.53). No patient relapsed within the predetermined 8-week follow up after initial cure. Daily number of bowel movements changed from a median (IQR) of 6 (5-10) and 7 (6-10) in the colonoscopy and NGT groups, respectively, the day prior to FMT (p=0.436) to 1 (1-1) and 2 (1-2) 8 weeks after the infusion (p=0.165, see Fig.2).

Secondary outcomes: Self-reported health rating using a standardized questionnaire scale of 1-10, with 1 being the lowest, and 10 being "your best recent health baseline" increased over the study period from a median (IQR) of 5 (3-6) and 4 (2-5) in the colonoscopy and NGT groups, respectively, the day prior to FMT (p=0.436) to 8 (7-10) and 7 (5-8) 8 weeks after the infusion. The colonoscopy group had consistently higher health scores, accounted for by a higher reported score at day -1. When analyzing the absolute increment in scores, the groups did not differ (p=0.51).

Adverse events deemed likely related included mild abdominal discomfort and bloating in 4 patients (20%). One child treated colonoscopically had a transient fever of 38.8°C on day 2 that resolved spontaneously. There were several serious adverse events which were assessed as unrelated by the investigators and IRB, and reflect the relatively poor health of many with recurrent CDI. One patient died 12 weeks after the procedure, while hospitalized secondary to an acute exacerbation of chronic obstructive pulmonary disease, including bleb rupture requiring intubation and chest tube. Although she was treated for several weeks with

parenteral broad-spectrum antimicrobials, her CDI did not recur. Another patient died of metastatic laryngeal cancer 21 weeks after the procedure. A third patient was diagnosed with adenocarcinoma of the esophagus. A fourth patient, treated by the upper gastrointestinal route, was hospitalized for Fournier's gangrene.

Fecal Microbiota: 14 stool samples from 4 donors and 65 samples from 19 recipients (21 pre-FMT and 44 at different time points post FMT) were analyzed. The Shannon Diversity Index of fecal microbiota obtained from recipients evaluated prior to FMT was consistently low (mean±SD, 2.52±0.77) and increased after FMT (3.82±0.74) to a diversity level comparable to that of the donors' (4.20±0.51, $p<0.001$ for the difference between pre and post FMT; $p=0.53$ for the difference between post FMT and donor stool) as shown in Figure 4. This level persisted over time, and there was no significant difference between the diversity index in stool samples obtained in the first week after the procedure and those obtained up to six months later ($p=0.11$; Supplementary Appendix). The route of administration made no difference in the mean Shannon Diversity Index obtained after FMT (3.79±0.64 in the colonoscopy group compared to 3.84±0.84 in the NGT group, $p=0.245$; Fig 5). The microbiota composition and trajectories after FMT can be viewed in Figures S3 and S4 in the Supplementary Appendix.

Discussion:

In this small randomized controlled feasibility study, we demonstrated that infusion of unrelated frozen donor stools is efficacious in treating patients with relapsing/recurring CDI with an overall cure rate of 90% at 8 weeks. Furthermore, NGT seems to be a viable route of administration for the inoculum, a distinct advantage in the elderly and debilitated patients that are prone to this condition who may not tolerate a colonoscopy or the sedation associated

with the procedure. These data are especially encouraging in view of our study population, consisting of patients with at least 3 recurrences of CDI or two episodes of CDI resulting in hospitalization, in which the reported cure rate with standard antimicrobial treatment falls to <30% [7]. Moreover, 95% of patients had been treated with previous prolonged vancomycin tapers and 70% of participants had been treated with fidaxomicin in the past, even further lowering the likelihood of obtaining cure with standard antimicrobial treatment. One 89 year old patient with refractory disease had 16 documented episodes of CDI in the preceding 15 months, including 4 regular admissions and 2 admissions to the intensive-care unit. She was cured with two inocula and has been asymptomatic off treatment for 12 months.

Interestingly, of the two treatment failures noted in our study, one patient refused a second treatment dose after the initial inoculum had no curative effect. Unbeknown to us, we later learned that this patient self-administered homemade fecal enemas daily for a week, using unprocessed stool from his roommate. He subsequently reported feeling well and being completely asymptomatic, but as per our study definitions he was considered a treatment failure. This example also brings to light the potential hurdles associated with regulating a readily available “biologic therapeutic”, as can also be evidenced by numerous “how to” manuals published on the internet.

Though most of our patients were elderly, reflecting the main population in whom CDI develops, the mean age of our participants was only 54, influenced by the fact that we included three children in our study. This inclusion is important in view of the recent increase in the number of pediatric cases of CDI, including a growing population of children with

recurrent/refractory disease [1,32,33]. All three pediatric patients were cured after administration of a single inoculum.

Since completing administration of FMT to the 20 study subjects, we have performed an additional 11 “expanded access” clinical administrations of FMT using frozen inocula from unrelated donors with a success rate of 90.9%. All were delivered via NGT.

While a previous study demonstrated the superiority of FMT over standard antimicrobial treatment [21], the authors examined instillation of fresh donor stools. This strategy has several potential disadvantages, including the need for maintaining a readily available pool of donors, maintaining updated medical screening of donors, and, finally, the challenging logistics of obtaining the stool sample, processing the inoculum and delivering the FMT within a limited timeframe. The use of frozen inocula addresses many of these obstacles by allowing identification and screening of donors ahead of time and establishment of a bank of pre-processed and vetted material that is a readily-available on short notice. The banking of donor stools also allows the added safety of following donors for a period of time and retesting for infectious diseases that could potentially have been latent at the time of donation, prior to administration of the inoculum. The optimal “shelf life” of the inoculate is still unclear, but in our study the longest an inoculum was stored prior to clinical use was 156 days (mean 79.3 days).

Despite numerous reports of successful resolution of CDI by FMT, the treatment has yet to become an available therapeutic option for many patients. This lack of availability not only deprives patients of the potential benefits of the procedure, but encourages patients to seek unregulated sources of information and alternative FMT providers, leading to treatment with

unscreened fecal materials. As mentioned, this limitation can be partly explained by the logistical hurdles associated with the procedure [34]. Another inhibiting factor is the fact that the available data are mostly based on retrospective case series and include only a single randomized trial [21,24,25] to date, making practitioners cautious about adopting FMT as a viable treatment alternative. The variability in patient population, donor selection, inoculum preparation and route and volume of administration all make pooling of published results challenging. Our study protocol (attached in the Supplementary Appendix) may be of value in standardizing FMT, and we hope, if adopted by others, will make future outcome data comparable between institutions.

A major limitation of our study is the small sample size. Nevertheless, our results were comparable to those in the literature when using fresh donor stools. Of particular importance is the fact that delivery of the inoculum through the upper gastrointestinal tract seems to be comparable to that of colonoscopic delivery, thus eliminating the need for sedation, anesthetic risks, and colonic "clean out." Possible vomiting and aspiration is a concern with upper GI delivery, although we did not observe this complication in our study subjects or in 11 subsequent cases for care. We have now addressed this concern in part by further concentrating and encapsulating this inoculum in Capsugel DR hypromellose capsules, which resist dissolution in acidic environments. We are now studying oral delivery of frozen encapsulated material as the next logical step in making FMT more accessible to patients.

In conclusion, in our initial feasibility study, FMT using a frozen inoculum from unrelated donors was effective in treating relapsing CDI, even in patients with multiple recurrences. NGT administration appeared to be as effective as colonoscopic administration.

Acknowledgements:

The authors would like to thank Dr. Ramnik Xavier and CSIBD (P30DK043351) for support of microbiome analysis.

Ilan Youngster received career support from Harvard Catalyst, The Harvard Clinical and Translational Science Center, funded by the National Center for Research Resources and the National Center for Advancing Translational Sciences, National Institutes of Health Award 8UL1TR000170-05 and financial contributions from Harvard University and its affiliated academic healthcare centers. The content is solely the responsibility of the authors and does not necessarily represent the official views of Harvard Catalyst, the National Institutes of Health, Harvard University, its affiliated academic healthcare centers, or its corporate contributors.

The microbiome analysis was funded in part with Federal funds from the National Institute of Allergy and Infectious Diseases, National Institutes of Health, Department of Health and Human Services, under Contract No.HHSN272200900018C.

Disclosures:

Mark Smith is on the Board of Directors of OpenBiome a 501c3 non-profit aimed at expanding access to fecal microbiota preparations by providing screened, ready-to-use fecal material for clinical use.

Dr. Hohmann is the receipient of a sponsored research award from Seres Health, Cambridge MA, to MGH for a clinical trial related to treatment of C. difficile colitis.

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Table 1. Select baseline characteristics of study population stratified by treatment group.

	Initial Colonoscopy	Nasogastric Tube	*p*-value
[a]Age (years)	50.4±28.8	58.6±19.6	0.739
[b]Female gender	6 (60)	5 (50)	1.00
[b]Time since initial CDI (months)	7 (3-34)	12 (3-66)	1.00
[b]Hospital-acquired CDI	2 (20)	3 (30)	1.00
[c]Number of CDI recurrences prior to FMT	4 (2-7)	5 (3-16)	0.42
[b]Previous vancomycin taper	9 (90)	10 (100)	1.00
[b]Previous use of fidaxomycin	5 (50)	7 (70)	0.64
[b]Hospital admissions in the past due to CDI	6 (60)	7 (70)	1.00
[b]Inpatient at time of FMT	2 (20)	3 (30)	1.00
[c]Number of BM 1 day prior to FMT	6 (4-13)	7 (5-13)	0.43
[c]Health status 1 day prior to FMT	5 (2-7)	4 (1-10)	0.21

[a]mean±SD, [b]n(%), [c]median (range). CDI – *Clostridium difficile* infection; FMT-fecal microbiota transplant.

Figure 1: Enrollment and Follow-Up

Figure 2: Number of bowel movements per 24 hours in study population.

Legend: Shown are mean number of daily bowel movements (BM) in both study arms. Baseline represents reported BM prior to contracting *C. difficile* as reported by the patients. 6-month follow up is missing data from 3 patients in the NGT group and 2 patients in the colonoscopy group.

Figure 3: Reported health status over time in study population

Legend: Shown are mean scores of subjective well-being over time as reported using standardized questionnaire with a scale 1-10, 1 being the lowest. The colonoscopy group had consistently higher scores, accounted for by a mean higher reported score at day -1. When analyzing the absolute increment in health scores, the groups do not differ (p=0.51). FMT=fecal microbiota transplant.

Figure 4: Microbiota Diversity in Patients Before and After FMT, as Compared with Diversity in the Donors.

Legend: Shown is microbiota diversity in fecal samples obtained from fecal microbiota transplant recipients before and after the procedure, as compared with the donors, expressed by the Shannon Diversity Index. The box-and-whisker plots indicate interquartile ranges (boxes), medians (red horizontal lines), and range (whiskers). FMT=fecal microbiota transplant; SDI=Shannon Diversity Index

Figure 5: Microbiota Diversity in CDI Patients after FMT, Comparing Initial Route of Administration.

Legend: Shown is microbiota diversity in fecal samples obtained from fecal microbiota transplant recipients before and after the procedure, stratified by treatment route and expressed by the Shannon Diversity Index. The box-and-whisker plots indicate interquartile ranges (boxes), medians (red horizontal lines), and range (whiskers). CDI=*Clostridium difficile* infection; FMT=fecal microbiota transplant; SDI=Shannon diversity index.