Epidemiology and therapeutic possibilities of *Clostridioides difficile*, examination of the effectiveness of faecal microbiota transplantation

PhD thesis

by

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1. Introduction

1.1. The normal microbiota of human bowel

Gut flora or gut microbiota are the microorganisms including bacteria, archaea, viruses and fungi live in the digestive tract. They are permanent residents of humans with the highest concentration being found in the human colon. They coexist in close association with us, and most of them are important for the host. The microbiota has a great impact on nutrient degradation and adsorption. Recent studies found growing evidence for a relation between the human immune-, endocrine- and nervous system and intestinal microbiota.
The composition of the human bowel flora stayed unrevealed until recent times due to the inability to culture. With the use of culture-independent techniques, such as 16sRNA sequencing, the composition and the variation of the microbiota over time can be studied in great detail, such as the effects of antibiotic use on the stability of the gastrointestinal flora. It has been estimated that the human lower intestinal tract contains 100 x 10^{18} microorganisms belonging to between 5000 and 6000 species. The phyla *Firmicutes* and *Bacteroidetes* form the majority of the bowel microbiota followed by *Proteobacteria, Actinobacteria*, *Fusobacteria* and *Verromicrobia*.

Colonization resistance is the capacity of the normal flora to protect itself by limiting the invasion of exogenous and often harmful microorganisms. The host intestines' protection from exogenous pathogens was thought to result from microorganism-mediated direct inhibition. However, recent studies have shown that an indirect mechanism, known as immune-mediated colonization resistance plays an important role also. In stable conditions, pathogenic microbes do not have the opportunity to multiply and cause disease. When the normal gut flora is disturbed, particularly by antibiotic use, the colonization resistance is decreased, and the occurrence of pathogenic microorganisms related diseases is increased.

1.2. *Clostrioides difficile* infection

The first appearance of *Clostridioides difficile (*historically *Clostridium difficile)* in the scientific literature is dated in 1935 when HALL and O'TOOLE isolated a bacillus named *Bacillus difficilis* from meconium end faces of infants, described as a part of the bacterial flora. The species was transferred from the genus *Clostridium* to *Clostridioides* in 2016. It

was just in 1969 when its pathogenic potential was discovered by HAMMERSTROM et al. In their experiments the mono-contamination of germ-free rats by *Clostridioides difficile* often led to the development of transient diarrhea and occasionally to the death of the animals. The significance of this finding and the importance of the bacterium as a cause of disease in man remained unclear until the mid-1970s. In 1974, TUDESCO et al. in the United States, described an association between the development of pseudomembranous colitis (PMC) and perceiving antibiotic treatment by clindamycin. BARTLETT et al in 1977 isolated an unidentified *Clostridium* species from hamsters having a clindamycin induced colitis. This germ was confirmed as *C. difficile* and being responsible for the symptoms. In the same year, Larson et al. demonstrated that a cytotoxin can be detected in the stool of patients with histologically confirmed pseudomembranous colitis. The germ itself is an obligate anaerobic, spore-forming bacillus with a length of 3-5 μm. Under Gram staining it can be found predominantly positive, although older colonies can show Gram stain variability. On blood-based agar typically we note opaque, non-haemolytic colonies which have a diameter of 3-5 mm and an irregular or lobate edge. Sporulation occurs when the normal development of the bacteria is disturbed, and the cells receives environmental signals that vegetative growth is impossible. This sporulation cannot be detected on most *C. difficile* selective media.

The incidence of *C. difficile* infections (CDI) has increased to epidemic proportions over the past decades worldwide. Between 1996 and 2003, the prevalence of CDI in the USA doubled, reaching 61 per 100 000, and the rate of CDI listed as any diagnosis of hospitalized patients rose from 3.82 per 1 000 discharges in 2000 to 8.75 per 1 000 discharges in 2008. Clinicians noticed a larger proportion of severe and recurrent cases than previously reported. The increasing incidence of such infection can be partly explained by the spread of fluoroquinolone-resistant strains belonging to the PCR- ribotype 027, the production of binary toxin. Epidemics caused by ribotype 027 strains have been recognized in hospitals in many European countries. In 2012 a pan-European point prevalence study on healthcare associated infection, organized by the European Centre for Disease Prevalence and Control (ECDC), showed that 48% of gastrointestinal infections in the hospitals were caused by *C. difficile* and 7,7% of all health-care associated infections were due to CDI. Data from Germany proved that then incidence of healthcare associated CDI is 2-4 times higher than the incidence of nosocomial infections caused by methicillin-resistant *Staphylococcus aureus* MRSA. In Hungary the first appearance of the ribotype 027 was described in 2007 and by consequence the number of CDI cases increased rapidly. In 2010 30.4% of the 601 *C. difficle* isolate was found to be ribotype 027 and this proportion rose to 50.2% of 699 isolates in 2011. In 2014 192 toxogenic *Clostridioides difficile* strains were collected in two regions of Hungary. The most frequent ribotybes identified by capillary gel electrophoresis were 027 (33.3%), 036 (19,7%), 014 (6,7%) and 176 (6.7%). This latter is closely related to ribotype 027. We performed a retrospective cohort study at Pécs. Data were collected from electronic medical records and supplemented with chart review. All patients treated for *C. difficile*–associated diarrhea during hospitalization on the Infectious diseases ward of University of Pécs from January 2009 to December 2014 were identified. Rates of CDI have been increasing at our department also. It was unique as a given diagnosis in 2009, it occurred in 271 of our 1047 patients (25.9%) in 2013. In this six-year period 6218 hospitalization was made, of whom 886 (14.2%) cases were CDI associated. From the total of 886 cases, 493 (55.6%) were female and 393 (44.4%) males. The mean age of the

patients was found to be 70.1 years with the range of 26-92 ears. The average number of recurrent episodes was 2.16 and the proportion of severe cases was 66%.

An alteration of the gut microbiome allows the occurrence of colonization with *C. difficile* if the patient is exposed to the organism or its spores. The bacterium *C. difficile* produces several properties that contribute its virulence. The two most important virulence factors seem to be the spore formation and the toxin production. Sporulation occurs when the normal development of the bacteria is disturbed, and the cells receives environmental signals that vegetative growth is impossible. Spores are thereby infectious and highly resistant. The pathogen can be isolated from the different surfaces of hospital types of equipment long after the patients have been treated for *C. difficile* infection. The most important toxins being produced be the pathogenic strains of *C. difficile* are the toxin A and toxin B (also known as TcdA and TcdB). Historically toxin A was considered as an enterotoxin and toxin B as a cytotoxin, but recent studies suggest that toxin B has also an enterotoxic activity.

Exposure of the human colon to *C. difficile* toxins generates cell rounding and shedding of cells from the basement membrane, induced by loss of actin filaments. Shallow ulcers on the mucosal surface can appear and can be followed by the flow of serum proteins, mucus and inflammatory cells outward from the ulcer, creating the typical colonic pseudomembrane. This latter occurs by inspection as yellow or off-white raised plaques. Their usual diameter is between 0.2 and 2 cm and they are scattered over a fairly normal appearing intervening mucosa.

CDI can appear as a wide spectrum of clinical manifestations ranging from asymptomatic carrier state to fulminant colitis with megacolon or even perforation.

As CDI is a major public health problem an effective prevention of the disease should be primordial to reduce the number of cases and to prevent the poor or fatal outcome of the patients having a severe form. It is well known that optimal infection control practices and an antibiotic stewardship can significantly reduce the CDI incidence and transmission. However more effective preventive approaches require appropriate identification of patients at risk to optimize the cost-effectiveness of such interventions and the feasibility of the clinical trials. Some CDI risk factors are commonly reported, such as advanced age, co-morbidities, exposure of antibiotics, use of proton pump inhibitors (PPIs), histamine-2 receptor antagonists (H2RA) and exposure to health care settings. There are some other risk factors implicated such as obesity, non-steroidal anti-inflammatory drugs, vitamin D and the role of host genetics in acquiring CDI.

There is a variety of available options for laboratory testing to detect the *Clostridioides difficile*. Toxigenic culture (TC) is one of the reference methods against which other methods are compared. The other reference method is the cell cytotoxicity neutralization assay (CCNA), which detects toxin directly from the stool. Enzyme immunoassays replaced these methods for routine clinical testing. They use monoclonal or polyclonal antibodies to detect *C. difficile* toxins. Glutamate dehydrogenase immunoassays (GDH) are the initial screening tests in 2- and 3 – step algorithms. They detect a highly conserved metabolic enzyme, which is present in all isolates of *C. difficile.* The nucleic acid amplification tests (NAATs) appeared

in the early 1990s. They detect a variety of gene targets including *tcdA*, *tcdB* and 16S ribosomal RNA. These assays are more sensitive than toxin EIAS, but they are less sensitive than TC. Recent recommendations suggest that use of stool toxin test as a part of a multistep algorithm (i.e. GDH plus toxin, GDH plus toxin arbitrated by NAAT or NAAT plus toxin) should be considered if there are no preagreed institutional criteria for patient stool submission. If the patient likely to have CDI based on clinical symptoms, the use of NAAT alone or a multistep algorithm can be applied for testing.

According to the recommendation of the European Society of Clinical Microbiology and Infectious Diseases (ESCMID), if a treatment is needed, in the case of initial episode of CDI fidaxomicin 200 mg twice daily is recommended for ten days as an initial treatment. When the access to fidaxomicin is limited, oral vancomycin 125 mg four times a day could be an alternative. In the cases of severe and severe-complicated CDI treatment options include vancomycin 125 mg four times daily for 10 days or fidaxomicin 200 mg twice a day for 10 days. For the recurrent episodes of CDI, if the initial episode was treated with metronidazole or vancomycin, then fidaxomicin 200 mg twice daily for 10 days is the preferred option. Fecal microbiota transplantation could be a rescue therapy for patients having severe complicated CDI that have deteriorated despite CDI antibiotic treatment.

2. Fecal microbiota transplantation

The idea of Fecal Microbiota Transplantation (FMT) is to transfer of fecal material containing the whole intestinal flora and natural antibacterial (e.g.: antibodies) from a healthy individual into a patient, who suffers from *Clostridioides difficile* infection. Other terms for the procedure include fecal bacteriotherapy, fecal transfusion, fecal transplant, stool transplant, fecal enema, and human probiotic infusion (HPI). The first transplantation in humans was reported in 1958, Eisemann et al. have treated four critically ill patients with fulminant pseudomembranous colitis using fecal enemas and, in all patients, symptoms resolved within hours of FMT.

Currently, there is little data available to suggest who might be an ideal donor. Clinicians often choose individuals living in the same household (e.g., spouse, child, parent) to act in the role of a donor, hypothesizing within intimate contacts, the microbiota may have already been widely shared by both parties, which minimizes the risk of transmitting any infectious agent. Neither the exact amount of donated feces needed for the transplantation nor the volume of fluid has been clearly determined; meta-analyses suggest a larger volume of feces will increase the success rate and reduce the relapse rate, since ideal clinical results seem to be greatest (97%) when more than 500 ml of suspension is transferred (while the resolution rate was found 80% when less than 200 ml is flushed in), and four times higher relapse rates have been reported when less than 50 g of stool is donated. It is highly considerable to eliminate antibiotics prior to FMT, however variances in the timeframe can be observed. Most centers stop administering antibiotics 1-3 days prior the procedure. Many authors suggest the usage bowel lavage. If FMT is to be delivered by naso-gastric tube, then a PPI should be given to the recipient the evening before and the morning of the procedure.

The first reported FMT in humans occurred in 1958 and was achieved via a retention enema. Since then, three main types of FMT delivery have been developed according to the location: proximal lower GI tract delivery- colonoscopy; distal lower tract delivery- enema and rectal tubes and upper GI tract delivery- nasogastric (NG) tubes, gastroscopy and duodenal tubes.

Each technique has its distinct advantages and disadvantages, and, in the end, the optimal procedure should be a patient-centered decision, based on both the wishes of the patient and the clinical aspects, e.g., the patient' s general condition, the available equipment at site, skills and previous experiences of the medical staff.

3. Aims and outcomes

The excessive use of antibiotics led to the occurrence of multidrug resistant organisms and a dramatic elevation in the number of total cases of *Clostridioides difficile* infections (CDI). The proportion of severe cases is significantly elevating, and clinicians now have to contend with the problem of additional and more frequent episodes of recurrences, including an upward trend in the mortality rate.
The aim of this thesis was to report the efficacy of fecal microbiota transplantation (FMT) via upper gastrointestinal tract delivery with patients who did not respond to antibiotic treatment, or who have suffered from multiple recurrences.

During our work, we tried to find answers for the following questions:

- How changed the epidemiology of the *Clostridioides difficile* associated infection in our region
- Which could be the risk factors?
- How can we make the donor screening in a more cost-effective way?
- How can we standardize the preparation of the fecal specimen?
- Which preparation method is the most effective?
- Is the FMT could be effective via upper gastrointestinal tract installation?
- Which way is more effective, the use of naso-gastric tubes or the use of naso-jejunal tubes?
- Can we use solution for installation based on the dilution of lyophilized feces for FMT with the same outcome when using freshly prepared solution?
- To create the foundations for the development of a much more convenient transplantation method with capsules containing the transplant material.
- If this latter is effective, its efficacity is equal with the efficacity of other methods?

4. Materials and methods

A CDI patient was defined as an adult with at least three nonformed stool per day and positive test results for *C. difficile* toxin. Stool samples were collected at the department and were sent to the stool laboratory of the National Public Health and Medical Officer Service of Baranya County. At the first period only ELISA tests for detecting toxins and culture were available. Both examinations were performed by the laboratory in each of the cases. Later C. Diff Quik Check Complete® (TechLab Inc.) were introduced. This is a rapid membrane enzyme immunoassay for the simultaneous detection of *C. difficile* glutamate

dehydrogenase antigen and toxins A and B in a single reaction well. Culture was done only in those cases where the toxin assay had been found negative.

However, when we started our study, there was no clear evidence as to the exact indications of FMT, according to the latest European and American treatment guidelines the indications of fecal microbiota transplantation in our practice were the following:

- Fulminant pseudomembranous colitis (PMC) which does not respond to treatment in 48 hours, multi organ failure or a septic state

- Severe PMC, which does not respond to antibiotic therapy in 7 days

- PMC combined with toxic megacolon, when there is no possibility of surgical intervention, or it is refused by the patient

- The first and second recurrence after a severe PMC treated successfully with antibiotics

- The third recurrence after PMC treated successfully with antibiotics

- Chronic or non-responsive PMC which leads to protein-losing enteropathy

In our practice, each donor served as a volunteer and was initially screened by means of a questionnaire adapted to local circumstances to exclude potentially high-risk individuals (e.g., drug use, unprotected sexual behavior or a history of extensive travel and illness or antibiotic intake in the past six months). They underwent a physical examination and general laboratory screening tests. Donated feces were screened for parasites, entero- pathogenic bacteria and *C. difficile*, while the serum was screened for HIV-1 and HIV-2, EBV, CMV, hepatitis A, B and C and *Treponema pallidum*.

At the beginning our goal was to use freshly-prepared samples from donors who were relatives of the patients in each of the correlating case. In our clinical practice the donated feces, were collected and transported to our department on the very day of the planned FMT. A 60 g sample was homogenized in a mortar and suspended in 200 ml of normal saline (0.9%). The suspension was then filtered through 4x4cm sheets of sterile gauze into another container, and 100 ml of the filtrate was absorbed into a syringe. All of the steps of the preparation were performed in a laminated flow box, and sterile devices were employed. Within 6 hours following the collection of the feces by the donor, the solution was infused into the patient. Later the logistical barriers (the viability of freshly- prepared samples is around 6 hours, the length of screening precluding the use of FMT in acute situations) of the procedure inspired us to investigate the possibility of the use of pooled, standardized samples, such as lyophilized or encapsulated specimen for FMT.

Generally, antibiotic administration was halted 4 days prior to the procedure. In serious cases this period was reduced to 1 day. In each case, thorough purging was applied on the day prior to the intervention. The patients consumed nothing from midnight, and in the morning before the procedure they received a double dose of proton pump inhibitor (PPI) intravenously. This increases the chances of survival of the transplanted flora by reducing the gastric acid output. One hour prior to the transplantation, 20 mg of *metoclopramide* was administered intravenously, to prevent vomiting and to facilitate bowel movement in order to promote the delivery of the transplantation material to the colon.

In our department, we performed only one lower GI tract FMT via colonoscopy, the rest of the fecal transplantations were performed via upper gastrointestinal way.

5. Results and Discussion

We performed FMT with freshly prepared specimen on a total of 64 patients. In all 16 initial cases, where the solution was instilled through ND tubes, we noted that diarrhea had stopped within 24 h, as did all of the other symptoms. No recurrences occurred in this group. Following 1 week, it should be noted that diarrhea resumed in the case of a 70-year-old female who previously had eight microbiologically proven recurrences of CDI. Clostridial toxins could not be detected, and the bacterium could not be cultured from her stools after the FMT. Detailed gastroenterological examinations subsequently revealed a late onset of irritable bowel syndrome in this patient.

When the inoculum was flushed in through NG tube, the symptoms resolved in 43 (89.58%) cases within 24 hours, but all the patients improved within 48 h. In 79.17% (38) of these cases, we did not experience any recurrences for 6 weeks; however, in ten cases (20.83%), microbiologically confirmed CDI recurred. These patients were aged (over 75 years), with a severe underlying condition, and it is important to note that one of them had previously been given antibiotics to treat his urinary tract infection caused by multidrug-resistant organisms. After the second transplantation, seven of the ten patients were cured, and thus the secondary cure rate in this subgroup has been found to 93.75%. In our hands, the overall primary cure rate of upper GI tract FMT was 84.37%, whereas the overall secondary cure rate was 95.31%. It is important to note the recurrence rate appeared to be lower when the solution was instilled through ND tubes. Although the difference ($p = 0.3113$, using Fisher's exact probability test) between the two groups was statistically not significant, we consider it to be clinically important. Nausea was observed in one case but could be controlled with vitamin B6 and metoclopramide. No other side-effects or reactions were detected, and therefore we cannot support the view in which the "yuck" factor is a relevant obstacle.

Between August 2013 and June 2015, we performed FMT with lyophilized TM on a total of 19 patients, of whom 9 (47.36%) were females. The mean age was 67.73 years, with a range of 38-94 years. In 16 cases (84.21%), the patients had severe CDI at the time of the transplantation. Unfortunately, we lost one patient within two days after the transplantation, who was suffering from severe comorbidity (COPD, polyvascular disease) and who was in a very poor general condition succumbed two days following the procedure. Among the surviving 18 patients, 15 (83.33%) reported having a normal stool within 48 hours following the FMT. Two of the non-responders underwent a second transplantation, and following a second treatment the clinical resolution of the diarrhea was reported in both cases. Only one patient was found to be non-responder at all, and she was treated by an additional course of fidaxomicin, and clinical resolution of her symptoms was reported within 4 days. We experienced in which FMT via NG tubes, using a freeze-dried specimen has the primary cure of 83.33%, while the secondary cure rate was found to be 94,44%. As it was mentioned before the overall primary cure rate of upper GI tract FMT was 84.37%, whereas the overall secondary cure rate was 95.31%, so our results with freeze-dried specimen are equivalents. No serious adverse events or recurrences were observed. None of the patients reported vomiting within 24 hours of administration. Mild adverse events e.g., bloating, abdominal cramps and nausea were reported in only 3 cases (16.67%).

As it was mentioned before the “yuck” factor, the aversion of patients can be a limit of the use of freshly prepared material. This is the reason, why our group continued the search for methods could be more acceptable for our patients. Even though this study is focused on FMT using freshly prepared samples, we think the notion of our experience with encapsulated specimen can be interesting.

We performed FMT with capsules in 28 patients without any side effects. 16 of them received capsules containing lyophilized supernatant. Fifteen of these patients recovered after a single dose of capsules (93.75%). The history of the patients who did not respond to treatment prior to transplantation included 22 relapses. Relapses were seen in two cured patients and were successfully treated with fidaxomicin later on. One patient passed away during follow-up, the cause of death was a comorbidity unrelated to CDI. 12 other patients received lyophilized fecal sediment. 8 of them were cured after one treatment and no recurrence was reported. One patient recovered after another stool transplantation and one after fidaxomicin administration. The combined cure rate of FMT by capsules was 23/28 (82.14%) in our practice.

6. Conclusion

Treating CDI and mainly recurrent CDI is a therapeutic challenge for patients and physicians. Current treatment options include repeated or prolonged courses of antibiotics with limited success rates, and there is lack of alternative therapeutic options (immunoglobulin preparations from vaccinated cows, probiotics). Fecal Microbiota Transplantation has a high cure rate nearing 100% even administered by upper GI tract, and it is reported safe which supports the viability of its use.
During this study and our clinical work, we tried to compare the effectiveness of different delivery methods. For better comparability, we tried to ensure standardized conditions during the preparation of stool samples. This is one of the strengths of our clinical work.
We must note that the number of cases in each group, is still too low to draw firm conclusions, so we consider it important to carry out further clinical studies.
Despite deficiencies in the design of our work, our results seem to adequately support Fecal Microbiota Transplantation via NG tube administration as a rational treatment option for patients with recurrent or refractory CDI, even by using a lyophilized material.

7.1. List of publications as a first author, related to this topic

1. Vigvári, S. et al., 2019. Faecal microbiota transplantation for *Clostridium difficile* infection using a lyophilized inoculum from non-related donors: A case series involving 19 patients. ACTA MICROBIOLOGICA ET IMMUNOLOGICA HUNGARICA, 66(1), pp.69–78. IF: 1,086; Q3

2. Vigvári, S. et al., 2019. Experiences with faecal microbiota transplantation in *Clostridium difficile* infections via upper gastrointestinal tract. ACTA MICROBIOLOGICA ET IMMUNOLOGICA HUNGARICA, 66(2), pp.179–188. IF: 1,086; Q3

3. Vigvári, S. et al., 2018. Risk factors for *Clostridium difficile* infections in Baranya County, Southern Hungary. ACTA MICROBIOLOGICA ET IMMUNOLOGICA HUNGARICA, 65(2), pp.183–192. IF : 1,079; Q3

4. Vigvári, S. & Péterfi, Z., 2016. Advances in fecal micriobiota transplantation. In Superbugs: *Clostridium difficile* and *Klebsiella pneumoniae* recognition, prevention and treatment. pp. 19–38.

5. Vigvári, S. et al., 2015. Faecal microbiota transplantation in *Clostridium difficile* infections. INFECTIOUS DISEASES, 47(2), pp.114–116. IF: 1,551; Q2

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Total IF of publications as a first author: 4,802

7.2 List of other publications related to this topic

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3. Varga, A. et al., 2021. How to Apply FMT More Effectively, Conveniently and Flexible – A Comparison of FMT Methods. FRONTIERS IN CELLULAR AND INFECTION MICROBIOLOGY, 11. IF: 6,073; Q1

4. Varga, A. et al., 2018. Treatment options of *Clostridium difficile* infection: our latest experiences with faecal microbiota transplant. CLINICAL CHEMISTRY AND LABORATORY MEDICINE, 56(9), p.eA133-eA133.

Total IF of publications related to this topic: 10,173

7.3 Other publications

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2. Péterfi, Z., Nemes, Z., Vigvári, S., Szomor, Á., Kereskai, L., Kucsera, I., Tánczos, B., et al., 2011. Visceral leishmaniasis in an immunocompetent Hungarian adult patient. HEALTH (IRVINE), 3(1), pp.1–5.

3. Péterfi, Z. et al., 2010. Magyarországra behurcolt visceralis leishmaniosis újabb esete. EPIDEMIOLÓGIAI INFORMÁCIÓS HETILAP, 17(3), pp.29–30.

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