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Infrequent transmission of Blastocystis via deep-frozen faecal microbiota transplantation: results from a randomised double-blinded cross-over study

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Abstract

Background

Traditionally, *Blastocystis* has been a reason for exclusion from stool donation for faecal microbiota transplantation (FMT), although evidence from clinical studies is very limited. We recently analysed *Blastocystis* data from an irritable bowel syndrome (IBS) trial, evaluating whether FMT using material positive for *Blastocystis* DNA leads to transmission of the protist.

Methods

The quantitative PCR data of *Blastocystis* originate from a three-arm, randomised, double-blind, placebo-controlled crossover clinical trial that tested the clinical effects of FMT in 50 patients with IBS. The live faecal material used for FMT remained consistent throughout the study, having been prepared in a single batch by mixing stools from eight donors and then stored frozen at -80°C. The placebo was the same material that had been autoclaved before freezing. The transferred material was highly positive for *Blastocystis* ST3 DNA, yielding a mean threshold cycle of 22.2. A total of 100 FMT interventions were performed, with a mean of 11 stool samples regularly collected from each participant tested for *Blastocystis*.

Results

One case of potential transmission was noted among the 31 patients who were initially *Blastocystis*-negative and received live FMT. This patient developed high positivity for *Blastocystis* ST3 DNA nine weeks after FMT, and this positivity persisted without any relevant adverse effects. One additional patient had a single positive sample for an untypeable trace load (Ct = 38.6) at week 24 post-FMT, transmission being considered unlikely as all other stool samples were negative. Pre-existing long-term colonisations with various subtypes (observed in three subjects) were unaffected by live FMT.

Conclusions

The frequency of *Blastocystis* transmission from a PCR-positive live FMT was very low, and had no adverse clinical correlates. This may be related to the decreased viability of the protist, given that all faecal doses for FMT were frozen at -80°C for at least three months during quarantine. This observation has implications for relaxing the selection criteria for FMT donors.

INTRODUCTION

Faecal microbiota transplantation (FMT) is a potentially life-saving procedure restoring the recipient's dysbiotic gut microbiota using a faecal microbiome bulk transferred from a healthy donor. It has been

tremendously successful in treating *Clostridioides difficile* infection (CDI) where it has become a therapeutic standard for the second recurrence of the disease [1], and its applications are also being studied in other diagnoses [2, 3].

The microbiological screening of the prospective stool donor must be very strict. The reason is logical: no other medical procedure transfers comparably wide repertoire of microbes from the donor to the recipient. Transferring bulk microbiome of the stool is inherently burdened with the risk of infecting the recipient. The only defence against such transmission of unwanted pathogenic agents is to meticulously screen stool donors. This concerns not only select viruses, bacteria, and fungi, but also protists.

While there is a wide consensus on what protist pathogens are contraindication for becoming a stool donor (e.g., *Cryptosporidium* spp., *Giardia* spp., *Entamoeba histolytica*), there is a general lack of consensus as to whether *Blastocystis sp.* should be on such a list, and how it should be tested for. *Blastocystis sp.* (hereafter referred to by its genus name *Blastocystis*) is a Stramenopile of a non-negligible prevalence among healthy population. Its presence in the gut microbiome has been associated with an increased alpha diversity of the gut bacteriome [4, 5, 6], and also with indicators of cardiometabolic health and healthier diet [7]. It can be therefore viewed as an indicator of a beneficial gut microbiome composition.

The current criteria for donor selection are available in positional papers and recommendations; we identified 15 such papers [2, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21]. The papers are listed in Table 1. Two of them recommend *Blastocystis* testing with the intention of excluding positive donors [10, 18], whereas in contrast four explicitly took a stance against excluding subjects with *Blastocystis* from stool donation or do not list it among the contraindications for becoming a donor [15, 17, 19, 21]. The remaining papers do not mention *Blastocystis* explicitly, but they recommend testing in general, often under the general umbrella term of 'ova and parasites' which indeed silently includes *Blastocystis* if detected microscopically or by multiplex PCR panels.

Table 1
Published FMT guidelines and their attitude towards *Blastocystis* positivity in the stool donor

The FMT guidelines published by	**Reference**	***Blastocystis* or general parasitology testing that is required for the donor stool to pass safety screening**	**Notes**
A Joint Position Paper From the NASPGHAN and the ESPGHAN	[8]	"Ova and parasites, if travel history"	Among important references is [9]
A review referred to by the Position Paper above, [8]	[9]	"Ovum and parasite microscopic examination"	No explicit recommendation given for *Blastocystis*
International consensus conference on stool banking	[10]	Testing recommended	
Australian consensus statements	[11]	"... there is insufficient evidence to support the practice of testing or excluding donors based on the presence of these organisms [*Dientamoeba fragilis* and *Blastocystis hominis*]"	"The pathogenicity of these organisms, however, has never been established in humans, and some studies suggest they may in fact be associated with health."
Spanish societies	[12]	none	
Polish Society for Epidemiology and Infectious Diseases	[13]	"Stool testing for the presence of parasites"	No further specification of the testing extent or type.
Position paper of the Catalan Society of Gastroenterology	[14]	"Conventional parasite test Real-time PCR study or available technique (EIA, etc.) for ... *Blastocystis* hominis ..."	A later update on *C. difficile* infection brings no new information on parasites [35]
UEG working group consensus statement	[15]	*Blastocystis* testing recommended	Colonisation with *Blastocystis hominis* is not considered an exclusion criterion. However, the working group advises to monitor for the effects of transmission.
Romanian national guideline	[16]	"Stool ova, cysts and parasite analysis, including for Microsporidia"	

NASPGHAN = North American Society for Pediatric Gastroenterology, Hepatology, and Nutrition

ESPGHAN = European Society for Pediatric Gastroenterology, Hepatology, and Nutrition

UEG = United European Gastroenterology

The FMT guidelines published by	Reference	*Blastocystis* or general parasitology testing that is required for the donor stool to pass safety screening	Notes
Danish national guideline	[17]	Intestinal pathogenic parasites: *Entamoeba histolytica, Cryptosporidium parvum/hominis, Giardia lamblia*, Worms	*Blastocystis* is not listed among the exclusion criteria for donors.
Clinical Practice Guidelines for Fecal Microbiota Transplantation in Korea	[18]	Testing recommended (as a strong recommendation, low quality of evidence)	*Blastocystis* among the agents listed as contraindication for being a donor.
A review on FMT	[2]	*Blastocystis* was discussed, and the rationale behind the exclusion of *Blastocystis*-positive donors was disputed	
British Society of Gastroenterology (BSG) and Healthcare Infection Society (HIS)	[19]	Cites a study documenting transmission of *Blastocystis* via FMT [22]	No need to intensify donor screening for the organism.
The American Gastroenterological Association (AGA)	[20]	Not mentioned.	
International Donor Screening Working Group	[21]	Possibility of *Blastocystis* transmission via FMT graded as "definite", whereas the clinical impact graded as 0 points (= colonisation)	Points out recent evidence linking *Blastocystis* to healthier diets and improved cardiometabolic outcomes.
NASPGHAN = North American Society for Pediatric Gastroenterology, Hepatology, and Nutrition			
ESPGHAN = European Society for Pediatric Gastroenterology, Hepatology, and Nutrition			
UEG = United European Gastroenterology			

The reasons for excluding subjects carrying *Blastocystis* from FMT donations are not well grounded in scientific evidence. At the time when we planned the herein reported FMT trial, transmission of *Blastocystis* was only scarcely mentioned in the literature - this may itself indicate its limited clinical consequences. In line with this notion, Terveer et al observed no clinical correlates for the transmission of *Blastocystis* via frozen FMT from two donors to 8 of 16 FMT recipients [22]: these recipients were *Blastocystis*-negative prior to FMT and the post-FMT PCR signal suggested stable colonisation of the recipients' gut without adverse effects on the success rates nor new symptoms in the recipients. Inspired by that work, we conducted a simple *in vitro* culture experiment, demonstrating that freezing at

-80°C indeed dramatically reduces the viability of *Blastocystis* [23]. Bacteria, in contrast, have been documented to retain their viability after a freeze-thaw cycle of the material for FMT [24].

Recently, Bénard et al published a case series of two subjects who temporarily acquired *Blastocystis* after FMT of fresh stool positive for the same subtype (the most frequently observed ST3), but lost it thereafter; this transient infection also remained without clinical correlates [25]. The report brings valuable data on the comparison of culture results from fresh and frozen stool samples, again indicating a diminished viability of *Blastocystis* after freezing.

In the present work we aimed to ascertain the transmission rates of *Blastocystis* in a double-blinded randomised study using FMT in irritable bowel syndrome (IBS) where participants received repeated enemas with a single mixed stool graft that was positive for *Blastocystis* DNA, or autoclaved placebo.

PATIENTS AND METHODS

The background study

The data in the present study originate in a clinical trial of mixed donor FMT in irritable bowel syndrome (IBS) whose protocol was reported earlier [26]. Its primary endpoint was a reduction in IBS symptom severity score, and the secondary outcomes included the composition of gut microbiota, including the parasite fraction.

This was a randomised double-blinded cross-over placebo-controlled trial with three arms. Its design, along with stool sample timing for follow-up, is shown in **Supplementary Fig. 1**. Patients in the study arm A received first a pair of enemas with live microbiota, and after 8 weeks they received a pair of enemas with placebo (the same mixture but autoclaved), whereas in arm B the sequences of interventions was swapped (placebo first, then live microbiota). In addition to these, there was the arm C where patients received only placebo, administered in the same time points as in arms A or B. The study was approved by the institutional ethics committee, and all participants expressed their consent in writing. The study has been at clinicaltrials.gov (NCT04899869).

All patients were evaluated using questionnaires administered at predefined time points that assessed symptom scores of IBS and adverse events, and by regular faecal bacteriome profiling. The results of the primary and secondary study endpoints will be reported elsewhere. Adverse events were monitored throughout the study and classified by severity based on the guideline outlined in the Common Terminology Criteria for Adverse Events (CTCAE) version 5.0 [27].

Stool material for FMT, and its application

The stool donors for the mixed faecal material for FMT were selected based on their large bacteriome distance from faecal bacteriomes of patients with IBS, and on their high alpha diversity, as described in

the study protocol along with the criteria for donor screening [26]. The donor exclusion criteria did not include *Blastocystis* positivity. Out of seven stool donors, five were *Blastocystis* positive at least once during their longitudinal observation, which extended long beyond the stool donation for the present study. Two of donors were highly positive for the ST3 subtype of *Blastocystis* during the period of stool donation for the present study, whereas further three were trace-positive around the time when they donated, also for the ST3 subtype, as shown in **Supplementary Fig. 2**.

The donor stools were brought to the laboratory within 2 hours of defecation. There, they were frozen at -80°C for quarantine. After the quarantine period, the stool portions were thawed to 0–4°C in cold buffer and mixed together into a common pool under a nitrogen atmosphere. The resulting 8.3 kg (21 litre) pool was filtered and aliquoted into suitably sized doses, and frozen again at -80°C until administration. The FMT bulk was tested for *Blastocystis* using four different DNA extractions from various stored FMT material aliquots: positivity was noted in all, the threshold cycle being 21.5, 21.5. 21.6, and 24.0. Culture or microscopy of *Blastocystis* from the fresh donor faecal material was not performed.

The FMT was administered as enema, in two doses applied two days apart: the first dose was after a reduced bowel preparation protocol, whereas the other was administered natively. After administration, the patient was positioned to distribute the enema in the colon. The study was double blinded with placebo being the same mixed donor material frozen after having been autoclaved. The visual appearance of placebo was identical to that of the live FMT material.

Testing the recipients for Blastocystis

Every participant was instructed to collect 11 faecal samples at pre-specified time points according to the protocol. The patients received detailed written instructions to ensure standardized collection and storage procedures. Samples were obtained at home into a sterile tube using a spoon attached to the tube lid, placed into pre-frozen transport containers (both Sarstedt, Germany) and temporarily stored in domestic freezers. The containers were then used for the transport to the clinic, with samples being kept frozen. Upon receipt at clinical centre, samples were transferred to long-term storage at − 80°C. Faecal DNA (50–100 mg) was extracted using the DNeasy PowerSoil Kit on a QIAcube automated instrument (both Qiagen, Germany), following the manufacturer's instructions. The detection and quantification of *Blastocystis* was performed using internally controlled real-time quantitative PCR (qPCR) assay using the protocol originally described by Stensvold et al [28] with minor modifications - the workflow is described in detail in **Supplementary Methods**, **Blastocystisdetection**. All *Blastocystis* positive samples were subjected to subtyping using massively parallel amplicon sequencing according Maloney et al [29], i.e. with primers originally developed by Santin et al [30] extended with Illumina Nextera tails, and with spacers to increase the sequencing pool diversity [31] (**Supplementary Methods**, **Blastocystissubtyping**).

RESULTS

The patients at risk of Blastocystis transmission

The FMT trial comprised 50 patients who completed the study protocol. There were 18 patients in arm A (live microbiota, then placebo), 16 in arm B (placebo, then live microbiota), and 16 in arm C (placebo only). Thus, the latter 16 patients receiving placebo only were never exposed to live faecal material and therefore were not at risk for *Blastocystis* transmission by FMT. One of these subjects had a pre-existing high persistent positivity for *Blastocystis* throughout the study (the ST2 subtype). Further two had an isolated low positivity in one stool sample each (ST2 and an untypeable positivity).

The patients in arms A or B (34 subjects) received two consecutive FMTs with frozen-thawed live microbiota highly PCR-positive for *Blastocystis* DNA, and were thus at risk for *Blastocystis* transmission. The arms differed in the sequence of placebo and live FMT. Of these patients, 31 were negative at the start of the study, while pre-existing - and persistent - *Blastocystis* positivity was noted in three subjects: one was persistently positive for ST1 (patient no. 31), one for ST2 (patient no. 6), and one (patient no. 53) carried a combination of ST2 + ST3 at the study outset, and then ST3 since the first intervention, which was placebo. This is summarised in a flow chart in **Supplementary Fig. 3**, and all tested samples are plotted in **Supplementary Fig. 4**.

Transmission of Blastocystis

We noted two cases of possible transmission following to these 68 live FMT interventions in 34 subjects. Subject no. 8 became *Blastocystis* positive two weeks after live FMT (having an untypeable low quantity), and then the positivity re-emerged 8 weeks after live FMT as a high-to-moderate quantity of subtype 3, which persisted (Fig. 1, **facet A**). This was concluded as a likely transmission of *Blastocystis* from the FMT material: the nucleotide sequence of the acquired strain was present in the live FMT material, albeit not being the most prevalent variant; this indicates a possible expansion of a minority strain originating from the FMT (Fig. 2).

Subject no. 16 had a very low *Blastocystis* DNA quantity 24 weeks after the administration of live FMT, after being negative in three intervening samples (Fig. 1, **facet B**). The subtype of this positivity could not be determined due to a low DNA load, and it was therefore classified as an improbable, yet possible case of transmission.

The pre-existing *Blastocystis* positivity remained unchanged after live FMT (Fig. 1, **facets C and D**). Patient no. 6 retained his ST2 variant; similarly, the original subtype persisted also in patient no. 31 (ST1). Our data indicate that also patient no. 53 retained his original variant profile after live FMT, as he carried a ST3 variant very lowly prevalent in the live FMT, and his allelic ST3 profile remained unaffected by the FMT intervention (Fig. 2).

Clinical correlates of Blastocystis transmission

The two patients with possible FMT-related *Blastocystis* transmission did not report any adverse events. Several other study participants reported adverse events graded as 1 and 2 of the CTCAE scale, i.e., mild, self-limiting, or mild requiring non-invasive treatment. No adverse events of grade 3 or higher were noted, i.e. requiring invasive medication or hospitalization. Association of *Blastocystis* transmission with clinical parameters was not formally evaluated because of the paucity of the event.

DISCUSSION

Our results show the low frequency of *Blastocystis* transmission via FMT in a study using frozen-thawed faecal material pooled from donors preselected by their bacteriome composition and diversity. Only one case of likely *Blastocystis* transmission was noted following 68 FMT enemas performed in 34 subjects, and in addition one very low newly acquired positivity was classified as a possible, yet improbable, transmission. These two recipients did not report any adverse events associated with FMT.

Previous reports of Blastocystis transmission

This paucity of transmission is in line with the very low number of previous reports in the scientific literature regarding *Blastocystis* transmissions via FMT: only two papers could be found [22, 25]. The reason is at hand: the real clinical risk may be of very limited magnitude, so *Blastocystis* is mostly not tested after FMT, and even though transmission might be anecdotally observed, it is a clinically unremarkable phenomenon which does not warrant reporting.

Terveer reported 8 of 16 patients exposed to *Blastocystis* positive freeze-thawed FMT to become colonised [22]. The difference to our patients with IBS where transmission was exceptional may lie in the underlying diagnosis: Terveer et al examined FMTs in patients with recurrent *Clostridioides difficile* infection (CDI) who were pre-treated with vancomycin as part of the protocol. Still, in these rather ill subjects, no unfavourable association of *Blastocystis* infection was noted for two most important clinical outcomes: success rate as to the absence of CDI relapses in the next 2 months, or the number of CDI relapses.

Bénard et al reported three recipients of fresh *Blastocystis*-positive FMT, of whom two became temporarily colonised without any apparent clinical correlates, whereas the third, initially colonised with ST2, retained his strain and was not re-colonised with the ST3 type from the donor. Similarly to us, their patients had IBS.

Why excluding Blastocystis-positive donors may be wrong

The negative consequence of exclusion of *Blastocystis*-positive FMT donors are remarkable, as discussed in detail by Melchiorri et al [32]: they not only noted negative effects on the economy of the stool bank, but it also observed an increased donor attrition. By excluding donors colonized with a protist that is associated with increased gut microbiome diversity [4, 5, 6], we may inadvertently eliminate the

very donors whose microbiota provides recipients with the richest and most diverse repertoire of potentially beneficial bacteria.

The *Blastocystis* transmission, or lack thereof, from positive FMT may be substantially influenced by the viability of the protist. Deep freezing of the faeces during quarantine may have been instrumental in this respect as shown by other and us [23, 25]. The current emphasis on properly quarantining donor stools may actually result in the protist being killed or disabled. Thus, PCR signal of *Blastocystis* DNA does not equal to a transmissible organism. It may still be prudent to bar from stool donation those carriers whose *Blastocystis* DNA load is excessive, e.g. over the 95th percentile of quantity distribution. It is however presently impossible to compare quantities between laboratories and in time, as no internationally recognised quantitative standard is available. The only internationally recognised calibrator, the American Type Culture Collection 50608DQ, was not available at the time of writing.

One more caveat should be mentioned: one of the rarer human *Blastocystis* subtypes, the ST7, is probably pathogenic [33, 34]. Routine subtyping of donors is technically unfeasible, yet the ST7 is likely associated with gut symptoms that should be readily detectable by routine patient history taken by regular donor questionnaires.

Strengths and limitations

The strengths of the study can be seen in the longitudinal observation of donors, as well as recipients, in the detailed analysis of the subtyping data, and in the consistent use of a single-batch made FMT mixture for all interventions. The subtyping sequencing data were analysed in great detail using zero-radius operational taxonomic units, dissecting the subtypes for better traceability of possible transmissions.

The study has several limitations: first, we did not perform culture of *Blastocystis*, thus we cannot conclude on the transmission (or lack thereof) with certainty from culture-amplified samples. To overcome this, we analysed several samples before and after the suspected transmission events, as well as in the live FMT material, using shotgun metagenomic sequencing, but we failed to reconstruct the *Blastocystis* genomes, likely because our relatively low sequencing depth of several million reads per sample.

Conclusions

Our data may support the notion of allowing subjects with *Blastocystis* to donate stool for FMT. Transmission of the organism was an exceptional phenomenon, and it was not associated with adverse events.

Declarations

Technical information:

Funding Declaration: The study was funded by the INTEREXCELLENCE II programme of the Ministry of Education, Youth and Sports of the Czech Republic (grant no. LUC23165), COST Action CA21105 and by the Ministry of Health of the Czech Republic in cooperation with the Czech Health Research Council, project No. NW26A-NIVB; project No. LX22NPO5103, European Union: NextGenerationEU.

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Data availability: The data are available at the corresponding author upon a reasonable request.

Consent to Publish declaration: Not applicable

Ethics and Consent to Participate: This clinical trial was approved by the Ethics Committee of the Institute for Clinical and Experimental Medicine and Thomayer University Hospital, Czech Republic (G-18-26), and was registered at clinicaltrials.gov (NCT04899869). All participants provided informed consent with participation in writing.

Author Contribution: OC and JH conceived the present substudy. PK and JV led the background clinical trial, conducted FMT, observations and collected all clinical data. KH, AC generated laboratory data on Blastocystis and its subtypes. OC analysed Blastocystis data and drafted the manuscript. All authors revised the manuscript.

Competing Interest declaration: There are no competing interest.

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Figures

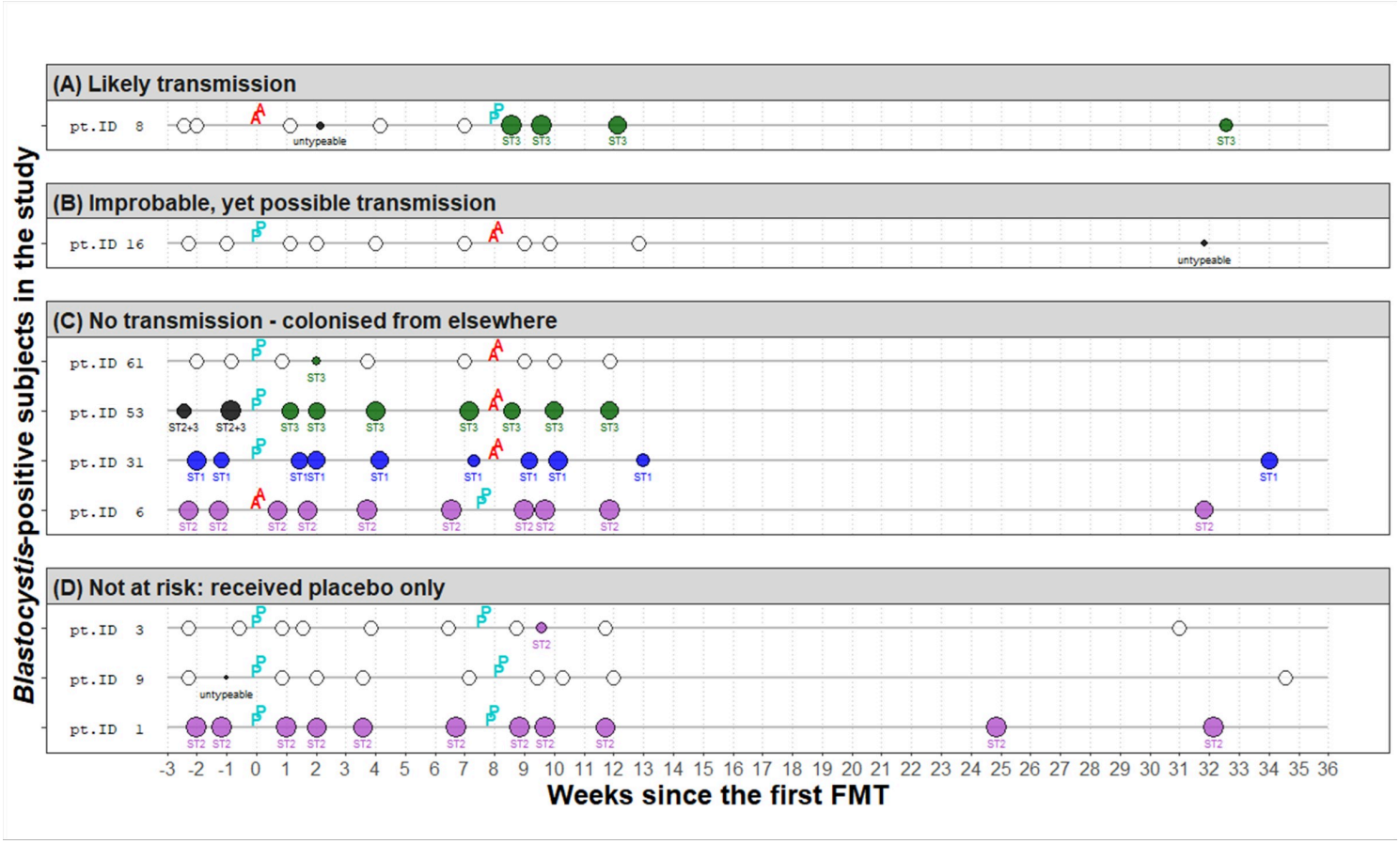


Figure 1

Patients having at least one sample positive for *Blastocystis*

A = FMT with active, live microbiota. P = FMT with placebo. The size of the symbol in positive samples is proportional to the logarithm of quantity.

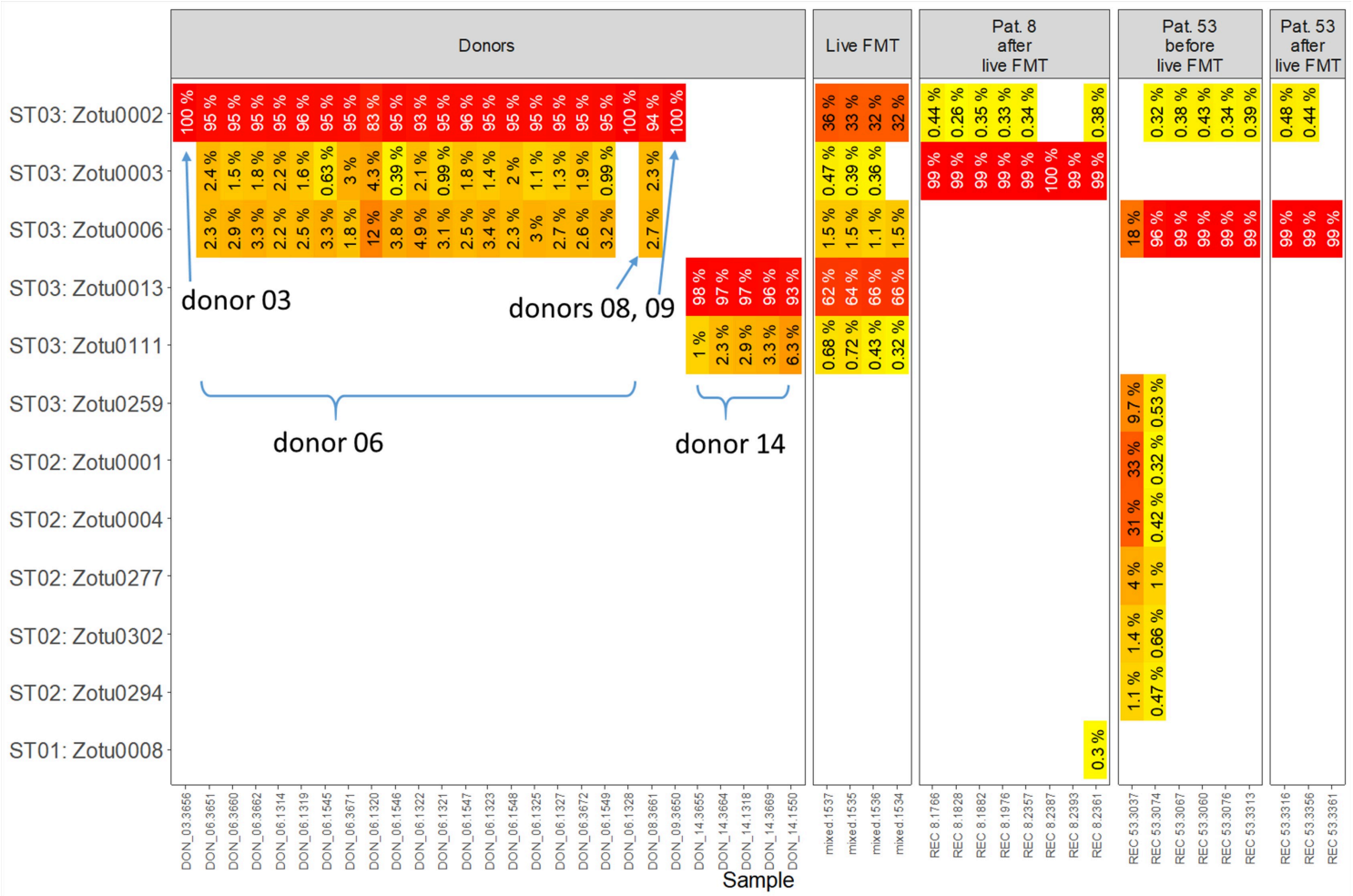


Figure 2

Profiling of the *Blastocystis* subtypes

Zotu = zero radius operational taxonomic unit; here it is a sequence variant of the 18S gene region used for subtyping of *Blastocystis*.

Numbers in cells are percentage of the variant among the total *Blastocystis* sequencing signal of the sample. Some of the minor values may reflect intra-organismal diversity of 16S copies rather than multiple *Blastocystis* strains in the same host.

The five donors positive for *Blastocystis* carried variants of the subtype (ST) 3. The first three variants, ZOTUs 0002, 0003 and 0006, differ very little: of their 412 nucleotides, there is a single nucleotide difference among 0002-0003 as well as 0003 and 0006, whereas the 0002 differs from 0006 by two nucleotides. The variants 0013 and 0111 are more distant (4-5 nucleotides) from the former three.

The profile of the mixed live FMT material is logically positive for ST3 only, having all five variant sequences represented.

Patient 8 was initially negative for *Blastocystis* DNA. After live FMT, he gradually developed positivity for ST3 variant that corresponds best to Zotu0003 - this is present also in the live FMT in a minute quantity. Thus, he may have acquired this sequence variant from the mixed material and expand it; the possibility of acquiring this variant from elsewhere should not be however discarded.

Patient 53 was positive for ST3 already long before live FMT. His first two samples contain a mixture of ST2 and ST3, whereas later the profile contains only ST3. The variant proportion profile showed no change after FMT, which makes a transmission event very unlikely.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- SupplementaryMaterial.pdf