

Culturomics: bacterial species isolated in 3 healthy donors for faecal microbiota transplantation in *Clostridium difficile* infection

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Summary

Background. *Clostridium difficile* infections are the main cause of nosocomial acquired diarrhea, because of prolonged antibiotic regimens. In the last years, mortality has increased due to recurrent infections caused by metronidazole and vancomycin resistant hypervirulent *C. difficile* strain 027. Faecal Microbiota Transplantation (FMT) is an infusion of faecal material obtained from healthy donors. This procedure reduces mortality in recurrent *C. difficile* infections (CDI). In this study we identified bacterial species obtained from donors' stool samples using culturomics.

Materials and methods. Three donors were selected for FMT in three recipients affected by CDI. Faces obtained for

FMT underwent culturomics, applying 17 different culture methods.

Results and conclusions. Several different bacteria were isolated from donors. In two donors 4 potentially pathogenic bacteria were isolated; this suggests the use of culturomics for donors' screening or for selection of bacteria to infuse.

Introduction

Human gut microbiota consists of approximately 10^{13} - 10^{14} bacteria (9). Its alteration seems to be involved in several diseases such as obesity, malnutrition, metabolic diseases and chronic inflammatory diseases. For this reason gut microbiota is considered like an organ, which in healthy humans confers metabolic role, deconjugation of bile acid, immunological effects and pathogens protection (15).

Clostridium difficile infection (CDI) is due to an alteration of microbiota homeostasis, following a long period of antibiotic therapy (6). Several intestinal complications could occur, such as recurrent CDI. Recurrent CDI is a recurrence of symptoms within days to several weeks after the first episode that occurs after discontinuation of antibiotic therapy. The risk of multiple episodes is high in patients who have already had one recurrence (5).

Faecal microbiota transplantation (FMT) is an infusion of faecal material obtained from healthy donors, we demonstrated its effectiveness in treating recurrent CDIs in a recently published clinical trial (3). Usually, after a screening to exclude microbial pathogens, feces of a healthy donor are used for FMT, without characterizing the composition of their microbiota.

In this study, we used a culturomic approach to isolate and identify the bacterial population in healthy donors' feces to be used in FMT in recurrent CDI patients.

Culturomics is a set of culture conditions originally applied to isolate bacterial populations considered unculturable. Culture conditions include: several enrichment broth media, blood culture bottles, culture on selective and non-

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selective agar media, different temperatures and times of observation from few days to months (8).

Materials and methods

All aspects of this study were approved by the Ethical Committee of “Fondazione Policlinico Universitario A. Gemelli”. Written consent was obtained for all participants.

Three faecal samples obtained from three healthy donors for FMT were analysed by culturomics.

Donors screening

The donors' health conditions were assessed as previously described (3). As regards faecal samples, they were tested for *C. difficile* Toxin A/B [Liaison, DiaSorin SpA, Saluggia (VC), Italy], enteric bacteria, protozoa and helminths, VRE (vancomycin-resistant Enterococci), MRSA (methicillin-resistant *Staphylococcus aureus*), and Gram-negative MDR (multi-drug-resistant) bacteria, according to international guideline procedures.

Donor 1 was a 45 years old daughter, donor 2 was a 42 years old daughter and donor 3 was a 43 years old brother-in law of each recipient.

Culturomics protocol

Thirty grams of each faecal sample were dissolved and re-suspended in 30 mL of saline solution. After the homogenization, there was a filtration step using patented filters, included in the Parasep® Faecal Parasite Concentrators kit (Apacor Limited, Sapphire Center, Wokingham, England). In these filters, large particles are rejected without occluding the 425µm pores, according to manufacturer's instructions.

The filtered 30 mL were divided into three aliquots of nearly 10 mL each and then centrifuged at 3500 g for 10 minutes. Supernatants were discarded and each one of the three pellets was resuspended: one in 5 mL of Brain Heart Infusion (BHI, Oxoid Deutschland GmbH, Wesel, Germany), one in 5 mL of supplemented Brucella Broth (BB, Remel INC., Lenexa, USA) and the last in 5 mL of rumen fluid.

Rumen fluid was obtained from a cow fed with alfalfa-hay, before slaughtering. We modified the protocol described by Santschi *et al.* (16); briefly rumen fluid was filtered through two layers of gauzes and it was stored refrigerated overnight at 4°C to settle down particulate material. Supernatant was submitted to filter sterilization through 0.22 µm Minisart® filters (Sartorius AG, Weender Landstr. Goettingen, Germany)

BHI suspension was divided into two aliquots of 2.5 mL each, which were inoculated into two bottles of paediatric blood cultures (Becton, Dickinson and Company, Benex Limited, Shannon, Ireland), four aliquots of 2.5 mL each were obtained from the other two suspensions inoculated into four bottles of anaerobic blood culture (Becton, Dickinson and Company, Ireland), which were incubated at 37°C, vortexed and then 100 µL plated on agar media at day 7 and day 14. Culture conditions are shown in Table 1.

Identification of isolated bacteria

Bacterial identification was performed by MALDI-TOF, Mass Spectrometry, on a Bruker MicroFlex LT (Bruker Daltonics, Billerica, MA, USA).

For plate extractions, 1 to 4 colonies from subcultures were spotted on the target plate. Each spot was overlaid with 1 µL of alpha-cyano-4-hydroxycinnamic acid (α -CHCA).

Spectra were acquired from 1000 laser shots at a minimum intensity threshold of 30 arbitrary units (A.U.). Plate calibration was performed on each run before spectral acquisition using a mixture of two proteins contained in Bacterial Test Standard (BTS) (Bruker Daltonics).

The BTS has been developed for the Quality Control (QC) process of the MALDI Biotyper system. Its specific composition covers the entire mass range of proteins used by MALDI Biotyper for precise identification of microorganisms, making BTS ideal for use with this mass spectrometry system. When BTS is used, MALDI Biotyper performs an automatic QC before each identification run. The QC process includes calibration of the mass spectrometer, a laser setting check and evaluation of spectrum quality. System performance is finally confirmed by achieving *Escherichia coli* identification, which must pass minimum

Table 1. Media and culture conditions.

1. Pre-incubation in paediatric blood culture bottle with rumen fluid and then 5% sheep blood, Chocolate and McConkey agar under aerobic, aerobe with 2.5% CO₂ and microaerophilic conditions at 37°C
2. Pre-incubation in anaerobic blood culture bottle with rumen fluid and then Columbia and Schaedler agar under anaerobic conditions at 37°C
3. Pre-incubation in paediatric blood culture bottle with Brucella broth and then 5% sheep blood, Chocolate and McConkey agar under aerobic, aerobe with 2.5% CO₂ and microaerophilic conditions at 37°C agar under aerobic conditions at 37°C
4. Pre-incubation in anaerobic blood culture bottle with Brucella broth and then Columbia and Schaedler agar under anaerobic conditions at 37°C
5. Pre-incubation under aerobic conditions in BHI and then 5% sheep blood, Chocolate and McConkey agar under aerobic, aerobe with 2.5% CO₂ and microaerophilic conditions at 37°C agar under aerobic conditions at 37°C
6. Pre-incubation in anaerobic blood culture bottle with stool filtered at 5 m and then Columbia and Schaedler agar under anaerobic conditions at 37°C
7. Pre-incubation in paediatric culture bottle with stool filtered at 5 m and then 5% sheep blood under aerobic conditions at 37°C
8. Pre-incubation in paediatric blood culture bottle with 5 mL BHI and then 5% sheep blood, Chocolate and McConkey agar under aerobic, aerobe with 2.5% CO₂ and microaerophilic conditions at 37°C agar under aerobic conditions at 37°C
9. Pre-incubation in anaerobic blood culture bottle with 5 mL BHI and then Columbia and Schaedler agar under anaerobic conditions at 37°C

performance criteria. The result is documented on each identification run report.

Plate calibration met acceptable specifications when at least seven of the eight proteins were detected within 150 ppm of the *in last fit* parameter, analysed with Flex Analysis software (Version 3.4; Bruker Daltonics).

Results

Following bacterial species were isolated from donor 1 *Actinomyces neuii*, *Actinomyces turicensis*, *Alistipes shahii*, *Bacteroides uniformis*, *Bacteroides intestinalis*, *Bacteroides salyersiae*, *Bifidobacterium adolescentis*, *Blautia coccoides*, *Clostridium aldenense*, *Clostridium scindens*, *Enterococcus faecium*, *Escherichia coli*, *Eggerthella lenta*, *Nocardia arthritidis*, *Parabacteroides distasonis*, *Parabacteroides johnsonii* and *Streptococcus anginosus*.

In donor 2 *Bacteroides intestinalis*, *Bacteroides thetaiotaomicron*, *Bacteroides uniformis*, *Citrobacter amalonaticus*, *Clostridium bifementans*, *Clostridium butyricum*, *Clostridium sordellii*, *Clostridium tertium*, *Enterococcus faecalis*, *Enterococcus faecium*, *Escherichia coli*, *Flavonifractor plautii*, *Lactobacillus rhamnosus*, *Parabacteroides distasonis* and *Pseudomonas agarici* were detected

Finally, in donor 3 we found *Bacteroides uniformis*, *Bacteroides vulgatus*, *Clostridium bifementans*, *Clostridium clostridioforme*, *Clostridium perfringens*, *Clostridium tertium*, *Enterococcus faecium*, *Escherichia coli*, *Klebsiella oxytoca*, *Parabacteroides distasonis* and *Proteus mirabilis* (Table 2).

Discussion

C. difficile is a Gram-positive, spore-forming, anaerobic pathogen, which is acquired via faecal-oral route, through ingestion of the bacterial spores. After reaching the anaerobic gut niche of susceptible patients, the spores germinate into metabolically active vegetative cells that produce two major exotoxins, Toxin A (TcdA) and Toxin B (TcdB) (19). Some strains of *C. difficile* produce a third toxin, CDT (*C. difficile* transferase), which may also be involved in the pathogenic process (1). These strains have been associated

with severe forms of disease in humans, even if the roles of these toxins in pathogenesis aren't clear yet (2).

Recurrent CDI is associated with prolonged antibiotic therapy that could destroy the normal intestinal flora, enhancing a decrease in colonization resistance (18). In fact the normal microbiota of adults is dominated by species of the phyla *Bacteroidetes* and *Firmicutes* (9), whereas the predominant species in patients with recurrent CDI were *Proteobacteria* and *Verrucomicrobia*, reducing dominance of *Bacteroidetes* (4).

The main knowledge about the composition of gut microbiota was acquired using molecular methods such as DGGE (11) and TGGE (12) analysis and recently metagenomics. For many years, several bacteria were considered non-culturable and so cultures were not used to study this microbiota, but in the last years, the cultural approach is having a rebirth with culturomics (8).

Culturomics is a laborious and time-consuming approach, which is considered very useful to study living bacteria.

Using culture methods, 90 new strains were isolated and several data were obtained about faecal bacteria in people with and without diarrhea. Langier *et al.* developed 18 culture methods (8) whereas Samb-Ba *et al.* 9 (17).

In this study we applied 17 cultural conditions at 7 and 14 days on faecal samples obtained from healthy donors for FMT. We found qualitative and quantitative diversity among the bacterial species isolated in donors; according to literature this is due to different socio-economic conditions, diet, geographic areas (5) and culture methods used (8). In fact, in absence of a standardized method, various culture conditions have been used: 212 or 18 (8) and 9 (17), with different numbers of bacterial species isolated for each sample. We were able to cultivate several strains of *Enterococci* different strains of *Bacteroides* and *Clostridia*. In donor 1 particular bacteria were detected: *Actinomyces neuii* implicated in human infections such as abscesses, ulcer infections, cellulitis, urinary tract infections, prostatitis, chorioamnionitis or invasive infections such as bacteraemia, prostheses infections, chronic osteomyelitis, peritoneal dialysis-associated peritonitis, chronic pericarditis, lymphadenitis and endophthalmitis (20); *Actinomyces turicensis* reported as a pathogen in endocarditis (7); *Eggerthella lenta* isolated in blood samples (10). *C. perfringens* was isolated in donor 3 and its pathogenic effects are well known: gas gangrene and food poisoning (14).

Table 2. Bacteria isolated from faecal samples of the three donors.

Donor 1	<i>Actinomyces neuii</i> ; <i>Actinomyces turicensis</i> ; <i>Alistipes shahii</i> ; <i>Bacteroides uniformis</i> ; <i>Bacteroides intestinalis</i> ; <i>Bacteroides salyersiae</i> ; <i>Bifidobacterium adolescentis</i> ; <i>Blautia coccoides</i> ; <i>Clostridium aldenense</i> ; <i>Clostridium scindens</i> ; <i>Enterococcus faecium</i> ; <i>Escherichia coli</i> ; <i>Eggerthella lenta</i> ; <i>Flavonifractor plautii</i> ; <i>Nocardia arthritidis</i> ; <i>Parabacteroides distasonis</i> ; <i>Parabacteroides johnsonii</i> ; <i>Streptococcus anginosus</i>
Donor 2	<i>Bacteroides intestinalis</i> ; <i>Bacteroides thetaiotaomicron</i> ; <i>Bacteroides uniformis</i> ; <i>Citrobacter amalonaticus</i> ; <i>Clostridium bifementans</i> ; <i>Clostridium butyricum</i> ; <i>Clostridium sordellii</i> ; <i>Clostridium tertium</i> ; <i>Enterococcus faecalis</i> ; <i>Enterococcus faecium</i> ; <i>Escherichia coli</i> ; <i>Flavonifractor plautii</i> ; <i>Lactobacillus rhamnosus</i> ; <i>Parabacteroides distasonis</i> ; <i>Pseudomonas agarici</i>
Donor 3	<i>Bacteroides uniformis</i> ; <i>Bacteroides vulgatus</i> ; <i>Clostridium bifementans</i> ; <i>Clostridium clostridioforme</i> ; <i>Clostridium perfringens</i> ; <i>Clostridium tertium</i> ; <i>Enterococcus faecium</i> ; <i>Escherichia coli</i> ; <i>Klebsiella oxytoca</i> ; <i>Parabacteroides distasonis</i> ; <i>Proteus mirabilis</i>

Since 2013, in our Institution FMT has been used to treat recurrent CDI. FMT includes: donor screening by clinical history, blood and faecal tests to be carried out 7 days before FMT, to reveal possible infections; laboratory activities to prepare feces in solution for the infusion in patients; endoscopic procedure (3).

On FMT day, the donor carries feces for infusion, which is to be made within 6 hours from the emission. Culturomics could be applied as an additional screening step to avoid to infuse even potential pathogens, such as those above described in donor 1 and 3.

In 2013, a pilot study showed that a *synthetic stool* solution with cultured bacteria could be an alternative to the use of donor faecal sample in the treatment of recurrent CDI (13).

Conclusions

In conclusion, culturomics could be a useful technique to set up an optimal *synthetic stool sample* which could have several advantages in terms of safety: i) known composition; ii) absence of potential infections acquired by donor during the period between screening and donation; iii) to have ready-to-use bacterial solutions for emergency infusions.

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