Research Article

Wei Hong**, Jing Yang*, Yumei Cheng, Xiaolin Huang, Fengqin Rao, Ting Zhang, Pixiang Wang, Jian Liao, Xiaolan Qi, Zhizhong Guan, Zhenhong Chen, Guzhen Cui

Bacteria co-colonizing with Clostridioides difficile in two asymptomatic patients

https://doi.org/10.1515/biol-2019-0071
Received March 11, 2019; accepted September 25, 2019

Abstract: Background: Clostridium difficile infection (CDI) is the leading cause of nosocomial diarrhea. Co-colonization of key bacterial taxa may prevent the transition from asymptomatic C. difficile colonization to CDI. However, little is known about the composition of key bacterial taxa in asymptomatic patients. Methods: In the present study, the culture method was used to examine the composition of stool microbiota in two asymptomatic patients from Guizhou, China. Results: A total of 111 strains were isolated and phylogenetic relationships were determined by 16S ribosomal gene sequencing and Molecular Evolutionary Genetics Analysis version 7. The results demonstrated that *Escherichia* (33.3%, 37/111), *Clostridium* (24.3%, 27/111) and *Enterococcus* (11.7%, 13/111) exhibited a high ratio in asymptomatic patients. These isolates derived from two phyla: *Firmicutes* (51.3%, 57/111) and *Proteobacteria* (44.1%, 49/111). In addition, co-colonization of human pathogens *Fusobacterium nucleatum*, *Ralstonia pickettii*, *Klebsiella pneumoniae*, *Klebsiella quasipneumoniae* and *Clostridium tertium* with C. difficile was identified. To the best of our knowledge, these pathogens have not been co-isolated with C. difficile previously. Conclusions: In summary, the present study identified the composition of fecal microbiota in two asymptomatic patients in Guizhou, China. These results suggested that co-infection with human pathogens may be ubiquitous during CDI progression.

Keywords: Clostridium difficile infection; asymptomatic patients; co-colonization; microbial diversity; 16S rDNA sequencing

1 Introduction

*Clostridium difficile*, recently renamed *Clostridioides difficile* [1], is a gram-positive, rod-shaped and strictly anaerobic human pathogen. *C. difficile* infection (CDI) is the leading cause of nosocomial diarrhea, which poses a major threat to health care facilities, including long-term care facilities, nursing homes and hospitals worldwide [2, 3]. The clinical symptoms of CDI range from mild diarrhea to pseudomembranous colitis, which may result in death.

The mechanism of CDI onset is associated with antibiotic usage. Antibiotics are used to treat bacterial infections; however, they disrupt the integrity of the intestinal microbiota in the human gut. The niche created by antibiotics provides a competing advantage to *C. difficile* against probiotics, thus leading to the propagation of *C. difficile* and overproduction of toxin A and toxin B [4]. Toxin A (enterotoxin) and toxin B (cytotoxin) induce cell death, inflammation and the accumulation of neutrophils, which result in various symptoms of CDI [4].

*Corresponding authors: Wei Hong*, Key Laboratory of Endemic and Ethnic Diseases, Guizhou Medical University, Ministry of Education, Guiyang 55004, Guizhou, China, E-mail: hongwei_2015@hotmail.com

Fengqin Rao, Ting Zhang, Xiaolan Qi, Zhizhong Guan*, Key Laboratory of Endemic and Ethnic Diseases, Guizhou Medical University, Ministry of Education, Guiyang 55004, Guizhou, China.

Wei Hong, Fengqin Rao, Ting Zhang, Xiaolan Qi, Zhizhong Guan*, Key Laboratory of Medical Molecular Biology, Guizhou Medical University, Guiyang 55004, Guizhou, China.

Jing Yang, Guizhou Mother and Child Health Hospital, Guiyang 550004, Guizhou, China.

Yumei Cheng, Department of Critical Care Medicine, the Affiliated Hospital of Guizhou Medical University, Guiyang 550004, Guizhou, China.

Xiaolin Huang, Jian Liao, School/Hospital of Stomatology of Guizhou Medical University, Guiyang 550004, Guizhou, China.

Pixiang Wang, Department of Biosystems Engineering, Auburn University, Auburn, Alabama 36849, United States

Zhenhong Chen, Guzhen Cui, School of Basic Medical Science, Guizhou Medical University, Guiyang 550025, China Guizhou 550025, Guizhou, China

*These authors contributed equally to this work.

ORCID: Wei Hong: 0000-0002-3317-9401; Guzhen Cui: 0000-0002-6500-039X
Following a course of antibiotic therapy for CDI, the recurrence of CDI has been described in 10-30% of patients after first infection and up to 60% after multi-episode infections [5]. Furthermore, recurrent CDI (RCDI) leads to increased morbidity and mortality [6], thus, the treatment of RCDI is still challenging. In recent years, fecal microbiota transplantation (FMT), which transfers healthy fecal microbiota from a healthy donor to a patient with RCDI, has been demonstrated to be effective in treating RCDI with an effective rate of ~90% [6, 7]. These results suggested that the integrity of the gut microbiota be key for the treatment of CDI and RCDI.

By using whole metagenome shotgun sequencing, Vincent et al. demonstrated that co-colonization with key bacterial taxa may prevent the increased proliferation of C. difficile [8]. In clinical practice, a number of asymptomatic patients with C. difficile colonization do not develop CDI. The present study hypothesized that the presence of certain microbes in these patients may serve a pivotal role in preventing the transition of asymptomatic colonization of C. difficile to CDI. Thus, these asymptomatic patients may be used as an appealing gut microbial homeostasis model in the nosocomial environment. Study of the composition of gut microbiota in this model may help develop treatments for CDI/RCDI. In addition, the intestinal microbial community in asymptomatic patients is easier to study compared with that of the healthy human fecal microbiome, as it contains lower bacterial diversity and retains pivotal information.

Although the gut microbiota composition of C. difficile asymptomatic carriers may be important for finding new CDI/RCDI treatment strategies, limited information is available regarding the bacteria that co-colonize with C. difficile in the asymptomatic patients. In developing countries, the awareness of CDI is insufficient, and the dietary habits are distinct from North America and Europe. The present study used the culture method to study the diversity of microbes in two C. difficile asymptomatic patients in Guizhou, China.

2 Methods

2.1 Selection criteria and ethics

Consecutive patients who were admitted to ICU wards of affiliated hospital of Guizhou Medical University between December 11, 2016 to August 25, 2017. These patients were screened for enrollment by following inclusion criteria, i) Patients were eligible for the study if they were receiving antimicrobial therapy and if their expected length of stay was more than 2 days; ii) patients who were willing to participate in the study. We recorded age, sex, reason for admission, and receipt of antibiotics. C. difficile infection (CDI) was defined as hospital-associated diarrhea (HAD) with a positive stool for C. difficile isolation. Asymptomatic patient was defined as a positive stool for C. difficile isolation without HAD [9].

**Informed consent:** Informed consent has been obtained from all individuals included in this study.

**Ethical approval:** The research related to human use has been complied with all the relevant national regulations, institutional policies and in accordance the tenets of the Helsinki Declaration, and has been approved by the Human Ethics Committee of Guizhou Medical University (approval no. 2017-004).

2.2 Sample collection and processing

Stool samples were collected in 50 ml DNase & RNase-free NEST® sample collection tube (Nest Scientific USA, Inc.) and transferred to the laboratory immediately on ice. The samples were soaked in the appropriate amount of fresh BHI medium for 10 min and vortexed for 10-20 sec. The mixed solution was serially diluted in fresh BHI medium and spread across BHI-blood or CCFA-blood (Cycloserine-Cefoxitin-Fructose Agar, Oxoid) agar [10]. The plates were incubated in an anaerobic chamber at 37˚C for 48 h [11]. Colonies were picked and further purified by re-streaking on a BHI-blood agar plate.

2.3 16S rDNA sequencing and phylogenetic analysis

The genomic DNA of purified strains were prepared using a TIANGEN® bacterial genomic DNA extraction kit (DP302; TIANGEN Biotech, Beijing, China). Primers for 16s-V4-515F (5’-GTGCCAGCMGCCGCGGTAA-3’) and 16S-V4-806R (5’-GGACTCHVGGGT-WTCTAAT-3’) were used to amplify partial 16S rDNA of isolated strains according to Lianbing Lin et al. [12]. PCR amplification was performed in a GeneAmp® PCR system 9700 (Applied Biosystems; Thermo Fisher Scientific, Inc., Waltham, MA, USA) using Q5® High-Fidelity Polymerase. The thermocycling conditions were as follows: 98°C for 30 sec; 30 cycles of 98°C for 10 sec, 55°C for 30 sec and 72°C for 10 sec; and a final extension at 72°C for 2 min. The PCR amplification products were recovered directly by TIANGEN® PCR.
purification kit (DP204, TIANGEN Biotech). A total of 20 μl molecular grade water (heated to 60°C prior to applying to the column) was used to elute purified 16S rDNA. The DNA samples were stored at -20°C prior to sequencing by GeneCreate Biotech (Wuhan, China). Sequencing primers were the same as the primers used in 16S rDNA amplification.

The sequencing results of both directions were assembled using SeqMan software of Lasergene (DNASTar, Madison, WI, USA). The partial 16S rDNA sequences were blasted against the 16SMicrobial database (ftp://ftp.ncbi.nlm.nih.gov/blast/db/v5) using the NCBI-blast-2.7.1 algorithm (ftp://ftp.ncbi.nlm.nih.gov/blast/executables/blast+/2.8.0alpha) and analyzed by Molecular Evolutionary Genetics Analysis version 7 (MEGA7) [13]. For phylogenetic analysis, the evolutionary history was determined using the Maximum Likelihood method based on the Tamura-Nei model [14]. Evolutionary analyses were conducted in MEGA7 [13].

Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach and then selecting the topology with superior log-likelihood value. The tree is drawn to scale. The analysis involved 23 nucleotide sequences. All positions containing gaps and missing data were eliminated. A total of 230 positions were identified in the final dataset. Evolutionary analyses were conducted in MEGA7 [13].

3 Results

3.1 Patient characteristics

A total of 51 patients were enrolled in the study during their hospitalization in the affiliated hospital of Guizhou Medical University from 11/12/2016 to 25/8/2017. Among these patients, one patient developed CDI; two patients were confirmed to exhibit asymptomatic C. difficile colonization (C. difficile was isolated from his/her stool samples, however, the patients did not develop any CDI symptoms, such as diarrhea and megacolon, Tables 1 and 2). The patient with CDI experienced diarrhea, which was not recurrent following antibiotic treatment. Patient characteristics are presented in Tables 1 and 2. The incident rate of CDI was nearly 2%, and incident rate of asymptomatic C. difficile colonization was nearly 4%.

3.2 Isolation of bacteria that co-colonize with C. difficile

The stool samples of asymptomatic patients were analyzed using BHIS-blood and CCFA-blood medium without antibiotics (Figure 1) [15, 16]. A total of 111 strains were isolated from the two fecal samples. The strains were purified, and the partial 16S ribosomal gene sequences were obtained and blasted against the 16SMicrobial database. The blast results are presented in Table 3. Among the strains, Escherichia species (E. marmotae and E. fergusonii; n=37; 33.3%) was the most abundant species co-colonizing with C. difficile. Enterococcus (E. saigonensis, E. faecalis, E. hirae; n=13; 11.7%) and Clostridium (C. clostridioforme, C. tertium; n=12; 10.8%) were ranked in the second and third place, respectively (Table 3).

3.3 Phylogenetic analyses

To determine the taxonomic associations of the isolated strains, a phylogenomic tree was constructed based on the values of nucleotide sequence pairwise similarity between the isolates. Strains from same species were clustered together (Figure S1). To provide an in-depth view of the association between isolated species, duplicated strains were omitted and the actual number of isolated strains was subsequently marked (Figure 2). A total of 22 types of strains were demonstrated to co-colonize with C. difficile. The strains were grouped into two major groups: Group 1 and group 2 (Figure 2). The majority of strains in Group 1 belonged to the Firmicutes phylum, whereas the majority of strains in group 2 belonged to the Proteobacteria phylum. Bacteria from the Bacteroidete phylum was not isolated in the two samples [8]. This may have been caused by the bias of the medium or the novel gut microbiota structure of the patients, which may be affected by specific dietary habits and antibiotic use.

Two types of C. difficile strains were isolated from the stool samples, which mostly related to the C. difficile JCM 1296 and C. difficile 630 strain (Figure S1). Among all strains, 51.3% (57/111) of the isolated strains belonged to the Firmicutes phylum, which contained three classes: Clostridia (57.9%, 33/57), Bacilli (40.4%, 23/57) and Negativicutes (1.8%, 1/55); 45.9% (49/111) of the isolates belonged to the Proteobacteria phylum, which contained four classes: Gammaproteobacteria (87.8%, 43/49), Proteobacteria (6.1%, 3/49), Alphaproteobacteria (4.1%, 2/49) and Betaproteobacteria (2%, 1/49). In the Fusobacteria phylum (4.5%, 5/111), Fusobacteriales was
Table 1. Patient clinicopathological characteristics

| Variable                          | Neither C. difficile infection nor colonization (n=51) | C. difficile infection (n=1) | C. difficile colonization (n=2) |
|-----------------------------------|------------------------------------------------------|-----------------------------|--------------------------------|
| Age, mean years (range)           | 56 (18-90)                                           | 71                          | 58                             |
|                                   |                                                      |                             | 70                             |
| Sex, Male (Female)                | 35 (16)                                              | 1 (0)                       | 1 (0)                          |
|                                   |                                                      |                             | 0 (1)                          |
| Duration of hospitalization, median days (range)
  a From admission until diagnosis of C. difficile infection or colonization (for patients with CDI and asymptomatic C. difficile colonization) or until discharge (for patients without infection or colonization). | 7 (5-21) | 8 | 6 | 6 |
| Reason for admission              |                                                      |                             |                                |
| Pneumonia                         | 17 (33%)                                             | 0                           | 1 (50%)                        |
|                                   |                                                      |                             | 0                              |
| Respiratory failure               | 4 (8%)                                               | 0                           | 0                              |
| Brain Injury                      | 12 (24%)                                             | 0                           | 0                              |
| Cerebral                          | 1 (2%)                                               | 0                           | 0                              |
| Pancreatitis                      | 2 (4%)                                               | 0                           | 0                              |
| Cholangitis                       | 1 (2%)                                               | 1 (100%)                    | 0                              |
| Gastrointestinal bleeding         | 4 (8%)                                               | 0                           | 0                              |
| Intestinal obstruction            | 2 (4%)                                               | 0                           | 0                              |
| Epilepsy                          | 1 (2%)                                               | 0                           | 0                              |
| Renal failure                     | 1 (2%)                                               | 0                           | 0                              |
| Myocardial infarction             | 1 (2%)                                               | 0                           | 0                              |
| Others                            | 5 (10%)                                              | 0                           | 0                              |
| Antibiotic usage b                |                                                      |                             |                                |
| Cefuroxime                        | 12 (24%)                                             | 0                           | 0                              |
| Cefoperazone sodium sulbactam sodium | 31 (61%)                                           | 1 (100%)                    | 0                              |
| Tinidazole                        | 14 (27%)                                             | 1 (100%)                    | 0                              |
| Meropenem                         | 13 (25%)                                             | 0                           | 0                              |
| Imipenem cilastatin               | 15 (29%)                                             | 0                           | 0                              |
| Vancomycin                        | 7 (14%)                                              | 0                           | 0                              |
| Fluconazole                       | 14 (22%)                                             | 0                           | 0                              |
| Mikafen                           | 11 (22%)                                             | 0                           | 0                              |
| Tigecycline                       | 10 (20%)                                             | 0                           | 0                              |
| Gentamicin                        | 1 (2%)                                               | 0                           | 0                              |
| Linezolid                         | 6 (12%)                                              | 0                           | 0                              |
| Levofloxacin                      | 2 (4%)                                               | 0                           | 0                              |
| Moxifloxacin                      | 3 (6%)                                               | 0                           | 0                              |
| Voriconazole                      | 6 (12%)                                              | 0                           | 0                              |
| Isoniazid                         | 2 (4%)                                               | 0                           | 0                              |
| Rifampin                          | 2 (4%)                                               | 0                           | 0                              |
| Ethambutol                        | 2 (4%)                                               | 0                           | 0                              |
| Pyrazinamide                      | 1 (2%)                                               | 0                           | 0                              |
| Ceftazidime                       | 1 (2%)                                               | 0                           | 1 (50%)                        |
| Cefmetazole sodium                | 1 (2%)                                               | 0                           | 0                              |
| Piperacillin-tazobactam sodium     | 4 (8%)                                               | 0                           | 0                              |
| Oxacillin sodium                  | 1 (2%)                                               | 0                           | 0                              |

b Antibiotic usage before confirming of CDI infection or colonization.
Table 2. Detection of *C. difficile* in patients with asymptomatic colonization and CDI.

| Patient ID | Sample type | Culture | Medication                        |
|------------|-------------|---------|-----------------------------------|
| Asymptomatic colonization |             |         |                                   |
| 37         | Stool       | Positive| Ceftazidime                        |
| 42         | Stool       | Positive| Piperacillin-tazobactam sodium     |
| CDI        |             |         |                                   |
| 41         | Stool       | Positive| Tinidazole & Cefoperazone sodium sulbactam sodium |

CDI, *Clostridium difficile* infection.

Table 3. Strain identification by the partial 16S ribosomal gene*.

| Strain                  | Strain isolated (Percentage of all isolates, %) | Phylum     | Class                | Score (Bits) | E Value |
|-------------------------|-----------------------------------------------|------------|----------------------|--------------|---------|
| *Escherichia marmotae*  | 33 (30)                                       | Proteobacteria | Gammaproteobacteria  | 387          | 2.00E-107 |
| *Clostridium difficile* | 15 (14)                                       | Firmicutes  | Clostridia           | 2102         | 0.00E+00  |
| *Clostridium clindamicoforme* | 11 (10)                                   | Firmicutes  | Clostridia           | 429          | 3.00E-120  |
| *Enterococcus saigonensis* | 5 (5)                                       | Firmicutes  | Bacilli              | 403          | 2.00E-112  |
| *Ruminococcus gnavus*   | 5 (5)                                         | Firmicutes  | Clostridia           | 420          | 2.00E-117  |
| *Enterococcus faecalis* | 5 (5)                                         | Firmicutes  | Bacilli              | 2193         | 0.00E+00   |
| *Escherichia fergusonii*| 4 (6)                                         | Firmicutes  | Bacilli              | 436          | 2.00E-122  |
| *Enterococcus hirae*    | 3 (3)                                         | Firmicutes  | Bacilli              | 771          | 0        |
| *Klebsiella pneumoniae* | 3 (3)                                         | Proteobacteria | Gammaproteobacteria  | 760          | 0.00E+00   |
| *Klebsiella quasipneumoniae* | 4 (4)                                     | Proteobacteria | Gammaproteobacteria  | 778          | 0.00E+00   |
| *Fusobacterium nucleatum* | 4 (4)                                       | Fusobacteriia | Fusobacteriales      | 422          | 6.00E-118  |
| *Ralstonia pickettii*   | 3 (3)                                         | Proteobacteria | Proteobacteria       | 403          | 2.00E-112  |
| *Bacillus tropicus*     | 2 (2)                                         | Firmicutes  | Bacilli              | 765          | 0        |
| *Shigella dysenteriae*  | 2 (2)                                         | Proteobacteria | Gammaproteobacteria  | 379          | 3.00E-105  |
| *Lactobacillus paracasei* | 3 (3)                                       | Firmicutes  | Bacilli              | 414          | 9.00E-116  |
| *Pseudochrobactrum lubricantis* | 2 (2)                                   | Proteobacteria | Alphaproteobacteria  | 418          | 8.00E-117  |
| *Blastia producta*      | 1 (1)                                         | Firmicutes  | Clostridia           | 436          | 2.00E-122  |
| *Veillonella parvula*   | 1 (1)                                         | Firmicutes  | Negativicutes        | 438          | 6.00E-123  |
| *Acinetobacter baumannii* | 1 (1)                                       | Proteobacteria | Gammaproteobacteria  | 773          | 0        |
| *Bacillus cereus*       | 1 (1)                                         | Firmicutes  | Bacilli              | 2141         | 0.00E+00   |
| *Fusobacterium simiae*  | 1 (1)                                         | Fusobacteriia | Fusobacteriales      | 427          | 7.00E-117  |
| *Herbaspirillum chlorophenolicum* | 1 (1)                                    | Proteobacteria | Betaproteobacteria  | 424          | 2.00E-118  |
| *Clostridium tertium*   | 1 (1)                                         | Firmicutes  | Clostridia           | 719          | 0        |

*Green represents strains that have been reported as normal human habitats and may act as protective taxa against CDI. Red represents human pathogens that co-colonize with *C. difficile*. Black represents bacteria of which the pathogenicity to human is unknown (except *C. difficile*).
Bacteria co-colonizing with Clostridioides difficile in two asymptomatic patients

633

asymptomatic patients with C. difficile colonization. A total of 111 strains were isolated from these samples, their partial 16S ribosome genes were sequenced, and NCBI-blast-2.7.1 and MEGA7 algorithms were used to determine the diversity and phylogenetic associations of these isolates. The isolates were derived from three phyla: Firmicutes, Proteobacteria and Fusobacteria. Firmicutes (51.3%) and Proteobacteria (44.1%) were most abundant phyla. To the best of our knowledge, this is the first time that Fusobacteria was co-isolated with C. difficile, which was not previously demonstrated in metagenomic research [8].

4 Discussion

The present study used the culture method to analyze the microbial diversity in the stool samples of two asymptomatic patients with C. difficile colonization. A total of 111 strains were isolated from these samples, their partial 16S ribosome genes were sequenced, and NCBI-blast-2.7.1 and MEGA7 algorithms were used to determine the diversity and phylogenetic associations of these isolates. The isolates were derived from three phyla: Firmicutes, Proteobacteria and Fusobacteria. Firmicutes (51.3%) and Proteobacteria (44.1%) were most abundant phyla. To the best of our knowledge, this is the first time...
that Fusobacteria (4.5%) was reported to co-colonize with C. difficile. Comparing with metagenomic research [8], Bacteroidete phylum was not identified in the present study, which may be due to the bias of screening medium and antibiotics used during the hospitalization of patients.

Although metagenomic sequencing approaches can provide abundant data for culturable and nonculturable microorganisms, culturomics has become increasingly important in recent years [17], as it may enable the design of a defined microbiota composition, which may be transferred to patients with CDI by FMT. Ann M.O’Hara et al. have suggested that the microbial composition of the gut contributes to intestinal disorders and that the enhancement of beneficial bacteria may represent a promising therapeutic strategy against various diseases (e.g., CDI) caused by disruptions in the gut microbiota [18]. The present study demonstrated that Escherichia (33.3%), Clostridium (24.3%) and Enterococcus (11.7%) exhibited high ratios in the two tested samples, which was consistent with previous research [19]. These species may serve as protective taxa against the transition from asymptomatic C. difficile colonization to CDI. For example, Clostridium spp. are potential protective bacterial taxa that may exert their protective effects through the production of secondary bile acids [8]; Lactobacillus paracasei strains have been demonstrated to exhibit health-promoting properties as probiotics [19, 20]. Recently, Blautia producta, Ruminococcus, Lactobacillus paracasei and Escherichia have been used in a defined stool substitute mixture to treat antibiotic-resistant C. difficile colitis [21].

By contrast, species that are normally considered human pathogens were also identified to co-colonize with C. difficile, including Fusobacterium nucleatum, Bacillus cereus, Shigella dysenteriae, Ralstonia pickettii, Klebsiella pneumoniae, Klebsiella quasipneumoniae and Clostridium tertium. Fusobacterium nucleatum normally colonizes in the oral environment and has recently been demonstrated to be associated with intestinal tumorigenesis [22].
**Bacillus cereus** is easily transferred through food and may cause emetic or diarrheal food-associated illness [23]. *Shigella dysenteriae* causes dysentery, which occurs most frequently in areas where poor sanitation and malnutrition are prevalent, especially in developing countries [24]. *Klebsiella quasipneumoniae* has been reported to cause pyogenic liver abscess [25]. *Clostridium tertium* commonly affects neutropenic patients with haematological malignancy [26]. In addition, to the best of our knowledge, *Fusobacterium nucleatum*, *Ralstonia pickettii*, *Klebsiella pneumoniae*, *Clostridium tertium* and *Klebsiella quasipneumoniae* have not been reported to co-infect with *C. difficile* [27]. These results suggested that co-infection may be ubiquitous during CDI progression. In this case, it could be associated to the special dietary habits in the Guizhou province, where pickled and spicy food is preferred. However, the underlying mechanism needs to be studied further.

In the two asymptomatic patients, *C. difficile* was detected by culturing method. Due to the number of asymptomatic patients in present study was limited and the microbiota composition is strongly influenced by their illness and medical treatment [8]. Therefore, we could not perform statistical analyses to assess general abundance of microbial taxa for asymptomatic patients. Furthermore, there are still two concerns should be carefully addressed in future studies. Firstly, the diversity of microbes was relatively low in the present study. For instance, *Bacteroidete*, *Virus* and *Fungi* were not identified; this may have been due to the bias of the screening medium and/or antibiotic use of the patients during hospitalization. These problems should be carefully addressed in future studies. Secondly, co-colonization may also increase the potential for genetic transference of resistance, which results in the development of antibiotic-resistant pathogens [28]. However, weather horizontal transfer of antibiotic resistance-associated genes occurs among the isolated species is largely unknown.

In summary, the present study used the culture method to analyze stool samples from two patients with asymptomatic *C. difficile* colonization in Guizhou province. This is the first report of microbial diversity in *C. difficile* carriers in southwest China, where specific dietary habits are prevalent, with a preference for pickled and spicy food. The results of the present study may improve the awareness of CDI among clinicians and provide new options for CDI treatment in southwest China.

**Acknowledgments:** This work was supported by the National Natural Science Foundation of China (grant nos. 31560318, 31601012, 31760318 and U1812403) and Program of Scientific and Technological Innovation Team of Guizhou Province [no. (2017)5652].

**Conflict of interest:** Authors state no conflict of interest.

**References**

[1] Lawson PA, Citron DM, Tyrrell KL, Finegold SM. Reclassification of *Clostridium difficile* as *Clostridioides difficile* (Hall and O’Toole 1935) Prévot 1938. Anaerobe. 2016;40 95-9.
[2] Warny M, Pepin J, Fang A, Killgore G, Thompson A, Brazier J, et al. Toxin production by an emerging strain of *Clostridium difficile* associated with outbreaks of severe disease in North America and Europe. Lancet. 2005;366 1079-84.
[3] Collins DA, Hawkey PM, Riley TV. Epidemiology of *Clostridium difficile* infection in Asia. Antimicrobial Resistance and Infection Control. 2013;2 21.
[4] Rupnik M, Wilcox MH, Gerding DN. *Clostridium difficile* infection: new developments in epidemiology and pathogenesis. Nature Reviews Microbiology, Published online: 2009;5:26.
[5] Kao D, Roach B, Silva M, Beck P, Rioux K, Kaplan GG, et al. Effect of Oral Capsule- vs Colonoscopy-Delivered Fecal Microbiota Transplantation on Recurrent *Clostridium difficile* Infection: A Randomized Clinical Trial. JAMA. 2017;318 (20):1985-93.
[6] Youngster I, Gerding DN. Editorial: Making Fecal Microbiota Transplantation Easier to Swallow: Freeze-Dried Preparation for Recurrent *Clostridium difficile* Infections. Am J Gastroenterol. 2017;112 (6):948-50.
[7] Youngster I, Russell GH, Pindar C, Ziv-Baran T, Sauk J, Hohmann EL. Oral, capsulized, frozen fecal microbiota transplantation for relapsing *Clostridium difficile* infection. JAMA. 2014;312 (17):1772-8.
[8] Vincent C, Miller MA, Edens TJ, Mehrotra S, Dewar K, Manges AR. Bloom and bust: intestinal microbiota dynamics in response to hospital exposures and *Clostridium difficile* colonization or infection. Microbiome. 2016;4 12.
[9] Han XH, Du CX, Zhang CL, Zheng CL, Wang L, Li D, et al. *Clostridium difficile* infection in hospitalized cancer patients in Beijing, China is facilitated by receipt of cancer chemotherapy. Anaerobe. 2013;24 82-4.
[10] Comparison of Cycleriserine-Cefoxitin-Fructose Agar (CCFA) and Taurocholate-CCFA for Recovery of *Clostridium difficile* during Surveillance of hospitalized Patients.
[11] George WL, Sutter VL, Citron D, Finegold SM. Selective and differential medium for isolation of *Clostridium difficile*. J Clin Microbiol. 1979;9 214-9.
[12] Lin L, Han J, Ji X, Hong W, Huang L, Wei Y. Isolation and characterization of a new bacteriophage MMP17 from *Meiothermus* Extremophiles. 2011;15 253-8.
[13] Kumar S, Stecher G, Tamura K. MEGA7: Molecular Evolutionary Genetics Analysis Version 7.0 for Bigger Datasets. Mol Biol Evol. 2016;33 (7):1870-4.
[14] Tamura K, Nei M. Estimation of the number of nucleotide substitutions in the control region of mitochondrial DNA in humans and chimpanzees. Mol Biol Evol. 1993;10 (3):512-26.
[15] Sorg JA, Dineen SS. Laboratory maintenance of *Clostridium difficile*. Curr Protoc Microbiol. 2009;Chapter 9.
[16] Chen YB, Gu SL, Wei ZQ, Shen P, Kong HS, Yang Q, et al. Molecular epidemiology of *clostridium difficile* in a tertiary hospital of China. J Med Microbiol. 2014;63:562-9.
[17] Lagier JC, Khelaifia S, Alou MT, Ndongo S, Dione N, Hugon P, et al. Culture of previously uncultured members of the human gut microbiota by culturomics. Nature microbiology. 2016;1:16203.
[18] O’Hara AM, Shanahan F. The gut flora as a forgotten organ. EMBO Rep. 2006;7(7):688-93.
[19] Hattori M, Taylor TD. The human intestinal microbiome: a new frontier of human biology. DNA Res. 2009;16(1):1-12.
[20] Bhatti MA, Frank MO. Veillonella parvula meningitis: case report and review of *Veillonella* infections. Clin Infect Dis. 2000;31(3):839-40.
[21] Petrof EO, Gloor GB, Vanner SJ, Weese SJ, Carter D, Daigneault MC, et al. Stool substitute transplant therapy for the eradication of *Clostridium difficile* infection: ‘RePOOPulating’ the gut. Microbiome. 2013;1(1):3.
[22] Kostic AD, Chun E, Robertson L, Glickman JN, Gallini CA, Michaud M, et al. *Fusobacterium nucleatum* potentiates intestinal tumorigenesis and modulates the tumor-immune microenvironment. Cell Host Microbe. 2013;14(2):207-15.
[23] Stenfors Arnesen LP, Fagerlund A, Granum PE. From soil to gut: *Bacillus cereus* and its food poisoning toxins. FEMS Microbiol Rev. 2008;32(4):579-606.
[24] Levine MM, DuPont HL, Formal SB, Hornick RB, Takeuchi A, Gangarosa EJ, et al. Pathogenesis of *Shigella dysenteriae* 1 (Shiga) dysentery. J Infect Dis. 1973;127(3):261-70.
[25] Breurec S, Melot B, Hoen B, Passet V, Schepers K, Bastian S, et al. Liver Abscess Caused by Infection with Community-Acquired *Klebsiella quasipneumoniae* subsp. *quasipneumoniae*. Emerg Infect Dis. 2016;22(3):529-31.
[26] Barakat M, Hernandez S, Benoît J, Pourshahid S, Mamoon Y, Martin GT. *Clostridium tertium*: An Unusual Cause of Pyogenic Liver Abscess. ACG Case Rep J. 2018;5:e30.
[27] de Graaf H, Pai S, Burns DA, Karas JA, Enoch DA, Faust SN. Co-infection as a confounder for the role of *Clostridium difficile* infection in children with diarrhoea: a summary of the literature. Eur J Clin Microbiol Infect Dis. 2015;34:1281-7.
[28] McKinley L, Becerra B, Moriarty H, Short TH, Hagle M, Reymann A, et al. Vancomycin-resistant *Enterococcus* co-colonization rates with methicillin-resistant *Staphylococcus aureus* and *Clostridium difficile* in critically ill veterans. Am J Infect Control. 2016;44(9):1047-9.
Supporting information

Figure S1. Phylogenetic tree of the 111 isolated strains that co-colonized with C. difficile in asymptomatic patients. The evolutionary history was determined using the Maximum Likelihood method based on the Tamura-Nei model [14]. Green represents strains that have been reported as normal human habitats and may act as protective taxa against CDI. Red represents human pathogens co-colonizing with C. difficile. Black represents bacteria of which the pathogenicity to humans is unknown (except C. difficile).