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Contents lists available at ScienceDirect

Asian Pacific Journal of Tropical Medicine

journal homepage: http://ees.elsevier.com/apjtm

Original research http://dx.doi.org/10.1016/j.apjtm.2016.06.003

Role of intestinal flora imbalance in pathogenesis of pouchitis

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ARTICLE INFO

Article history:
Received 15 May 2016
Received in revised form 16 Jun 2016
Accepted 21 Jun 2016
Available online 28 Jun 2016

Keywords:
Intestinal flora
Pouchitis
Bifidobacterium
Lactobacillus
Bacteroid

ABSTRACT

Objective: To discuss the role of intestinal flora imbalance in the pathogenesis of pouchitis.

Methods: The pouchitis rat model was established and the faeces sample and the mucous membrane sample were collected regularly, in which the bacterial nucleic acids were extracted for quantitative analysis of the intestinal flora in the samples through using the real-time quantitative PCR technique and high energy sequencing technology.

Results: The disorder phenomenon of the intestinal flora appeared at the 7th day of the experiment, and the pouchitis was presented at the 21st day of the experiment. At the 31st day of the experiment, compared to control group and non-pouchitis group, the quantity of *Bifidobacterium* and the *Lactobacillus* of the pouchitis model rats in the mucous membrane sample and the faeces sample were significantly decreased ($P < 0.05$), and the *Bacteroidetes*, *Faecalibacterium prausnitzii* and XIV *Clostridium leptum* subgroup in the mucous membrane of pouchitis were significantly decreased ($P < 0.05$). The IV *Clostridium coccoides* group was the main flora in the mucous membrane of pouchitis, the bacterial diversity of non-pouchitis group and control group was significantly higher than that of the pouchitis group ($P < 0.05$).

Conclusions: The intestinal flora imbalance is one of the factors that cause the incidence of the pouchitis; this study provides a clue of the pathogenesis and treatment direction of the intestinal inflammatory disease.

1. Introduction

The incidence rate of pouchitis of ulcerative colitis after the operation is 50%, and it will increase with the extension of time. There are 61% of the patients who are multiple attacked by this disease, and 5%–19% of the patients will develop into chronic pouchitis [1,2]. There are many similarities of the phenotype of the pouchitis, crohn disease and ulcerative colitis, which is that the individual predisposing gene is activated through the factors of infection and surroundings, and cause the abnormity of the tolerance of intestinal immune, hence leading to the continuous and sustained inflammatory reactions and damage of tissues [3–5].

Pouchitis is the intestinal inflammatory disease that can be expected, which is an uncommon model of intestinal inflammatory disease that can be follow-up researched from premorbid [6]. Therefore, pouchitis is seen as the research object to study and discuss the cause and effect relationships between the changes of the intestinal flora and the incidence of the inflammation, so as to not only providing new method and clue for the prevention and treatment of pouchitis, but also offering a clue to the pathogenesis study of crohn disease and ulcerative colitis. So far, the relationship between the intestinal flora imbalance and the pathogenesis of pouchitis is not clear yet. We establish pouchitis rat model, the expected intestinal inflammatory disease model to dynamically test the changes of

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Peer review under responsibility of Hainan Medical College.

Foundation project: It was supported by Natural Science Foundation of China (Grant Number: 81550049).

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intestinal flora through high-throughput sequencing and the Real-time PCR quantitative techniques to discuss its mechanism of action in the intestinal inflammatory reaction, and provide a clue of the pathogenesis and treatment direction of the intestinal inflammatory disease.

2. Materials and methods

2.1. Materials

QIAamp DNA Mini Kit and Real-time PCR were purchased from Qiagen Co., Ltd.; the extraction kits of bacterial genome DNA were purchased from Tiangen BioTech Co., Ltd. A total of 70 SPF grade male Lewis rats with body weight of 300–325 g were purchased from Animal Experiment Center of Shanghai Jiao Tong University. This experiment was approved by Ethics Committee of Jinling hospital, and all operations were strictly abided by the specifications of Regulation of Experimental Animals. The experimental operations were finished at Department of Comparative Medicine, Jinling Hospital, and the operation of gene detection was finished at State Key Laboratory of infectious disease diagnosis and treatment, First Affiliated Hospital of Medical School of Zhejiang University.

2.2. Methods

2.2.1. Establishment of experimental ileoanal pouch rat model

A total of 50 Lewis male rats with body weight of 300–325 g were fed in a simulation environment with constant temperature. The rats were fed with standard rodent's foodstuff in the metabolism cages of 5–7 d in order to adapt to the cages before the operation. They were fasted before 24–36 h of the operation, and their drinking water was replaced with 5% glucose solution to avoid the delay of the healing of anastomotic stoma caused by high catabolism state. The rats were anesthetized with intramuscular injection without using antibiotics before the operation, then the intestinum crassum was resected after opening operation, and ileoanal pouch was established to coincide with rectum, then the abdominal cavity was closed. The operations were all conducted in the aseptic conditions. The rats were put on the water jacket heating pad 24 h after the operation, then they were put into metabolism cages after 24 h for 4 d and fed with 5% glucose solution only; they were fed with rodent's foodstuff soaked with glucose at the 6th day of the operation, and fed with standard rodent's foodstuff after they had tolerance of the food soaked with glucose. A total of 20 SD normal male rats were selected as control group, and their general conditions were observed and recorded, and all rats were executed at the 31st of the experiment and the mucous membrane samples were collected. The faeces samples were taken out for detection every week, and the samples were all preserved in the temperature of −80 °C.

2.2.2. Extraction of DNA of mucous membrane and total bacteria in faeces

The cells were mechanical cracked by glass bead breaker, and DNA of mucous membrane samples and the faeces of rats were extracted referenced to CTAB method; the QIAamp DNA Mini Kit (Qiagen) was used to extract the DNA of bacteria in mucous membrane and the QIAamp DNA stool Mini Kit (Qiagen) was used to extract the DNA of the faeces. All operations were strictly abided by the product specifications, and all the DNA samples were preserved in the temperature of −20 °C.

2.2.3. 16S rRNA PCR analysis and 454 high-throughput sequencing of intestinal bacteria

The DNA of total bacteria in faeces samples was used as model for the PCR analysis on V6–V8 fragment of 16S rRNA gene sequences of the bacteria, and the primers of PCR were U968-GC and L1401 with GC clips. The DNA of total bacteria in mucous membrane samples and faeces samples was extracted as the model for the PCR analysis on V3–V5 fragment of 16S rRNA gene sequences of the bacteria, the 16S rRNA fragment V3–V5 variable region, which is the amplification substance of PCR, was sequenced by Sangon Biotech (Shanghai) Co., Ltd. through 454 high throughput sequencing method. The sequences after the 454 sequencing were effectively screened, and the screening criteria showed as follow: 1) at least one fragment of the sequence was complete matching with barcode sequence and the primer sequence; 2) the length of the sequence was over 50 bp; 3) the number of blurry bases of the sequence was not over 2.

2.2.4. Quantitative analysis of samples by real-time PCR instrument

The total bacteria, *Firmicutes*, *Bacteroidetes*, IV *Clostridium coccoides* (*C. coccoides*) group, XIV *Clostridium leptum* (*C. leptum*) subgroup, *Bifidobacterium* and *Lactobacillus* in the mucous membrane samples and faeces samples were quantitatively analyzed by ABI 7500 real-time PCR instrument. The total reaction system of PCR was 20.0 μL: 10.5 μLof SYBR Green Supermix, 0.3 mol/L of upstream primers and downstream primers, 1.0 μL of DNA sample and 7.8 μL of aquae sterilisata. The represented nucleic acid of *Escherichia coli*, *Bacteroides thetaiotaomicron*, *C. coccoides*, *C. leptum*, *Faecalibacterium prausnitzii* (*F. prausnitzii*), *Lactobacillus sobrius* and *Bifidobacterium longum* were respectively used as model to amplify their 16S rRNA gene, and make corresponding quantitative standard curve. The density of DNA of the PCR production was detected after the purification of the production, and calculated its copy numbers according to the formula: Copy = (C/X) × 0.912 × 1012. The using primers and the reactions of real-time PCR were showed in Table 1.

2.2.5. Data analysis and processing

After the experimental data were preliminary statistically processed by Excel 2007, and the SPSS16.0 software was used for the significant analysis between pouchitis group and ileoanal pouch group through the one-way analysis of variance method. Chi-square test and Logistic regression were used to analyze the change over time of structure and number of intestinal flora, respectively.

3. Results

3.1. General conditions and pathological changes of pouchitis

There was significant weight loss, fecal occult blood positive and diarrhea in 30 rats at the 21st day of the experiment, which means the model was established successfully according to the

Table 1
Real-time PCR analysis of the quantity of main functional bacteria flora in faeces sample at the first day (log copy numbers/g wet weight of samples).

Group	Objective bacteria						
	Total bacteria	*Bacteroidetes*	IV *C. coccoides* group	XIV a *C. leptum* subgroup	*F. prausnitzii*	*Lactobacillus*	*Bifidobacterium*
Control group	11.35 ± 0.25	9.26 ± 0.63	8.62 ± 0.41	9.69 ± 0.63	9.85 ± 0.26	7.79 ± 0.73	8.68 ± 0.27
Non-pouchitis group	11.64 ± 0.26	9.43 ± 0.74	8.57 ± 0.21	9.89 ± 0.35	9.86 ± 0.63	7.41 ± 0.47	8.74 ± 0.63
Pouchitis group	12.08 ± 0.31	9.13 ± 0.36	8.93 ± 0.37	9.14 ± 0.53	9.72 ± 0.29	7.08 ± 0.38	8.18 ± 0.37

criterion of shebani pouchitis model [7], and the 30 rats were rolled into pouchitis group, and the other 20 rats without the classical symptom in the model were rolled into non-pouchitis group. The general conditions of the rats in control group and non-pouchitis group were normal. And the symptoms of inflammations, such as loss of appetite, abdominal distension, less active, lusterless fur, sustained weight loss, diarrhea and with blood in stool occurred to the pouchitis group.

3.2. Quantitative analysis of main intestinal flora in faeces sample

The quantitative analysis of main intestinal flora in faeces sample was shown in Tables 1 and 2. As shown in Table 1, there was no significant difference in main functional bacteria flora in faeces samples among the three groups (Table 1). However, on the 31st day, compared to those of rats in control group, the quantity of total bacteria, *Firmicutes*, *Bacteroidetes*, IV *C. coccoides* group, XIV a *C. leptum* subgroup, *Bifidobacterium*, *F. prausnitzii* and *Lactobacillus* in the faeces sample of non-pouchitis group had no significant difference ($P > 0.05$). Compared to those of rats in non-pouchitis group and control group, the *Bacteroidetes*, IV *C. coccoides* group, XIV *C. leptum* subgroup, *F. prausnitzii*, *Lactobacillus* and *Bifidobacterium* in the pouchitis group were significantly different ($P < 0.05$), and the *Bifidobacterium* and *Lactobacillus* were significantly decreased ($P < 0.05$) (Table 2). The IV *C. coccoides* group was the main flora in the faeces sample of pouchitis with no inflammation, and the enterobacterium and *Streptococcus* were the main flora in faeces sample of non-pouchitis; the quantity of total bacteria had no significant differences ($P > 0.05$), but the bacterial diversity of non-pouchitis group and control group was significantly higher than that of the pouchitis group ($P < 0.05$).

3.3. Quantitative analysis of main intestinal flora in mucous membrane sample

The quantitative analysis of main intestinal flora in mucous membrane sample was shown in Table 3. Compared to the rats in control group on the 31st day, the results showed that the quantity of the total bacteria, *Bacteroidetes*, IV *C. coccoides* group, XIV a *C. leptum* subgroup, *Bifidobacterium*, *F. prausnitzii* and *Lactobacillus* in the faeces sample of non-pouchitis group was not significantly different ($P > 0.05$); compared to those of non-pouchitis group, the total bacteria, *Bacteroidetes*, IV *C. coccoides* group, XIV *C. leptum* subgroup, *F. prausnitzii*, *Lactobacillus* and *Bifidobacterium* of rats in pouchitis group were all significantly different ($P < 0.05$), and the *Bifidobacterium* and *Lactobacillus* were significantly decreased ($P < 0.05$); the IV *C. coccoides* group was the main flora bacteria in mucous membrane of pouchitis, and the enterobacterium and *Streptococcus* were the main flora bacteria in mucous membrane of pouchitis

Table 2
Real-time PCR analysis of quantity of main functional bacteria flora in faeces sample at 31st day (log copy numbers/g wet weight of samples).

Group	Objective bacteria						
	Total bacteria	*Bacteroidetes*	IV *C. coccoides* group	XIV a *C. leptum* subgroup	*F. prausnitzii*	*Lactobacillus*	*Bifidobacterium*
Control group	11.67 ± 0.61	9.13 ± 0.72	8.23 ± 0.26	9.85 ± 0.08	9.72 ± 0.31	7.89 ± 0.61	8.62 ± 0.31
Non-pouchitis group	11.89 ± 0.32	9.27 ± 0.82	8.37 ± 0.01	9.97 ± 0.16	9.83 ± 0.14	7.56 ± 0.52	8.75 ± 0.51
Pouchitis group	12.58 ± 0.73	6.14 ± 0.24$^{*\#}$	14.09 ± 0.25$^{*\#}$	5.72 ± 0.21$^{*\#}$	5.17 ± 0.21$^{*\#}$	4.13 ± 0.06$^{*\#}$	5.05 ± 0.09$^{*\#}$

$^{*}P < 0.05$ compared with the control group.
$^{\#}P < 0.05$ compared with the non-pouchitis group.

Table 3
Real-time PCR analysis of quantity of main functional bacteria flora in mucous membrane sample at 31st day (log copy numbers/g wet weight of samples).

Group	Objective bacteria						
	Total bacteria	*Bacteroidetes*	IV *C. coccoides* group	XIV a *C. leptum* subgroup	*F. prausnitzii*	*Lactobacillus*	*Bifidobacterium*
Control group	11.67 ± 0.61	9.13 ± 0.72	8.23 ± 0.26	9.85 ± 0.08	9.72 ± 0.31	7.89 ± 0.61	8.62 ± 0.31
Non-pouchitis group	11.89 ± 0.32	9.27 ± 0.82	8.37 ± 0.01	9.97 ± 0.16	9.83 ± 0.14	7.56 ± 0.52	8.75 ± 0.51
Pouchitis group	12.58 ± 0.73$^{*\#}$	6.14 ± 0.24$^{*\#}$	11.09 ± 0.25$^{*\#}$	6.72 ± 0.21$^{*\#}$	6.17 ± 0.21$^{*\#}$	5.13 ± 0.06$^{*\#}$	5.05 ± 0.09$^{*\#}$

$^{*}P < 0.05$ compared with the control group.
$^{\#}P < 0.05$ compared with the non-pouchitis group.

with no inflammation; however, the diversity of flora bacteria of non-pouchitis group and control group was significantly higher than that of the pouchitis group.

3.4. High-throughput sequencing

The results of high-throughput sequencing was submitted to NCBI, and through the BLAST search comparison and the software analysis, which showed that the diversity of intestinal flora bacteria in the faeces sample and mucous membrane sample of pouchitis group were significantly decreased ($P < 0.05$), compared to control group and non-pouchitis group.

4. Discussion

The pathogenesis of inflammatory bowel disease (IBD) is involved the interactions of many aspects, such as heredity, immune, intestinal flora and environment. Some clinical experiments have confirmed that improving the diversity of the intestinal flora will contribute to the effective treatment of the intestinal inflammation [8,9]. However, the species of the intestinal flora were numerous, and the sensibility and the specificity of traditional separation and identification method were not high, which can only quantitatively measure a part of the intestinal flora. 16S rRNA is a major parameter that is used for classification of microbial system in recent years. This kind of ribosome RNA of prokaryotic organism has both the conserved sequence and high variable sequence, and the PCR primer was used to amplify DNA according to the specificity of this genus design, which can be used for quantitative detection of the intestinal flora. The specificity and the sensitivity of the technical evaluation of PCR to the intestinal flora are higher than those of the traditional methods. According to the general trend, the application of 16s rRNA technique in the field of flora analysis will be more and more extensive.

Pouchitis is the common complication after the operation of ulcerative colitis, and the pathogenesis is still not clear [10,11]. Similar to the IBD, its pathogenesis process involves intestinal flora imbalance, but it is not clear that these changes are the initial alteration factors or the secondary changes of intestinal inflammation environment [12–18]. Real-time PCR technique was used to amplify 16s rRNA to dynamically monitor the changes of the intestinal flora of rats in pouchitis model and rats in control group during the process of construction, which confirm that the changes of intestinal flora happens before the incidence of pouchitis, indicating that the changes of intestinal flora might be one of the factors that cause the incidence of the pouchitis.

The previous research showed that the plate count of lactobacilli, *Clostridium perfringens*, *Bacteroides*, *Bifidobacterium* groups, enterococci and coliforms in pouchitis after the operation of ulcerative colitis had no significant differences [19], but the quantity of sulfate reducing bacteria in the faeces of the patients with pouchitis after the UC operation was significantly higher than that of the patients with no inflammation, previous inflammation and the patients who had received the treatment of antibiotics [20–22]. Another research of comparative before and after the treatment showed that the quantity of *Clostridium perfringens* and aerobe were significantly increased and the quantity of *Lactobacillus* and anaerobion were decreased during the period of activity of pouchitis [23].

The quantity of *Lactobacillus* and anaerobion were recovered to the normal standard after the inflammation was relieved through antibiotics treatment [24,25]. The same kind of research had also found that the quantity of *Lactobacillus* and *Bifidobacterium* during the period of activity of pouchitis was significantly decreased compared to the relieved period [26]. The results of this study had also proved this point.

Based on the previous research of 16s RNA molecular biological techniques, the fingerprinting techniques was applied to the comparison of the mucous membrane of pouchitis and the mucous membrane of ileoanal pouch and found that *C. coccoides* group was the main flora in the mucous membrane of pouchitis and the enterobacterium and *Streptococcus* were the main flora in the mucous membrane of ileoanal pouch [18]. The structure of intestinal flora of pouchitis was analyzed by another terminal restriction fragment length polymorphism technique (T-RFLPL) and found that IV *C. coccoides* group was the main flora in the mucous membrane of pouchitis [22]. It was found that the abundance of IV *C. coccoides* group in pouchitis model sample was significantly increased in this study.

One research of the treatment of intestinal flora of pouchitis through probiotics had found that the diversity of the flora in mucous membrane of pouchitis during the relieved period was increased and the diversity of fungus was decreased [25], which was consistent with what had been observed in this study. This study had found that diversity of the flora in non-pouchitis group and control group was significantly higher than that in the pouchitis group, the Proteobacterium in mucous membrane of ileoanal pouch was significantly increased, *Bacteroidetes*, *F. prausnitzii* and XIV *C. leptum* subgroup were significantly decreased, indicating that the intestinal mucosa inflammation might be negative related to diversity of the flora, and the decrease of the diversity of flora was happened before the symptoms of pouchitis, indicating that the decrease of the diversity of intestinal flora was one of the reasons that caused the pouchitis.

Although many researches had found that there were changes of many related flora and bacteria species in the intestinal inflammatory diseases, and even the relationships of some specific bacteria and the incidence of the diseases were confirmed, however, it was still not clear that these changes were the initial alteration factors of the incidence of intestinal inflammation or the secondary changes under the environment of the intestinal inflammation [27–30]. This study has confirmed that the changes of the intestinal flora happen before the intestinal inflammation and it is not the secondary changes, which is not only significant for illuminating the pathogenesis of the intestinal inflammatory disease, but also provides a clue of the pathogenesis and treatment direction for the intestinal inflammatory disease.

Conflict of interest statement

We declare that we have no conflict of interest.

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