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Detection and identification of *Prevotella Melaninogenica* in saliva samples of patients with ulcerative colitis

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Abstract

Background: Ulcerative colitis (UC) is a long-term inflammatory bowel disease (IBD) caused by abnormal immune responses, leading to inflammation and scarring in the large intestine. The bacteria *Prevotella melaninogenica*, found in the intestine and mouth, may contribute to UC. This study focuses on the detection of *P. melaninogenica* in the saliva of UC patients and compares them with the healthy control (HC) group.

Methods: The present study was a case-control study including 40 UC patients and 40 healthy controls (HCs) with an average age of 43.0 3± 10.3. This study used a real-time PCR test to investigate the frequency and average number of *P. melaninogenica* from the 16S rRNA gene sequence of *P. melaninogenica* in both groups.

Results: *P. melaninogenica* was more frequent in UC patients (77.5%) than HCs (45%) (p = 0.003). The patient group had more bacteria (339.31 ± 1082.29) than HCs (61.29 ± 154.03) (p = 0.005). Women in the UC group had more *P. melaninogenica* (492.35 ± 1427.61) than the control group (56.98 ± 123.50) (p = 0.0342). Similarly, men in the UC group (262.85 ± 664.97) had more bacteria than the control group (72.62 ± 222.76) (p = 0.015).

Conclusion: The current study showed that dysbiosis in *P. melaninogenica*, a bacterium in human saliva, could be important in the development of UC. Further investigation is needed to evaluate its use as a potential biomarker in the UC.

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Introduction

Ulcerative colitis (UC), a chronic idiopathic inflammatory bowel disease (IBD) of the colon, results in a continuous, variable-intensity superficial mucosal inflammation that extends from the rectum to the more proximal colon. Relapsing and remitting symptoms are UC's defining features (1). The defining signs of UC are tenesmus, rectal urgency, and bloody diarrhea (2). The incidence of UC has been rising in Asia, especially in industrialized countries (3). The incidence of UC in 2020 was between 9 and 20 cases per 100,000 individuals annually (4). In a study conducted in Iran, Olfatifar et al. anticipated that between 2021 and 2030, there would be a 2.5-fold increase in the number of common cases of IBD (5).

According to studies, rather than being primarily due to genetic variances, ethnic and racial disparities are more closely tied to environmental factors, dietary preferences, and lifestyle choices. Population-based research has not revealed any appreciable gender disparities in UC. The incidence peak for UC is in the second and third decades of life, followed by a second high rate between 50 and 80 (6,7). Several epidemiological clues suggest that an imbalance in gut microbiota may be responsible for the development of Inflammatory Bowel Disease (IBD). Dysbiosis refers to the dysregulation of the immune system's reaction to bacterial antigens due to changed commensal bacterial population composition (8). Characterizing the dysbiotic state present in the gut microbiome of UC has been the focus of several studies. These studies have reported a decrease in bacterial diversity, a decrease in the relative proportions of Enterococcus and Bacteroides, a member of Clostridium subcluster XIVa, and an increase in several opportunistic pathogens (9). In addition, UC has also been linked to several extraintestinal symptoms, such as oral lesions, which can appear concurrently with intestinal symptoms or prior to the onset of the disease and may even be the main presenting symptom (10). IBD patients typically exhibit various oral symptoms, including aphthous stomatitis, oral ulcer, and pyostomatitis vegetans, suggesting a connection between these manifestations and the oral microbiota. The etiology of gastrointestinal disorders such as IBD may be influenced by the ectopic intestinal colonization of a specific oral bacterium (11-13). *Prevotella* is the largest genus in the oral cavity, and one of the major phyla is Bacteroides (14). *P. melaninogenica* resides in the oral cavity and colonizes the mucosal surfaces of infants in the first few months of life. Also, a high concentration of *P. melaninogenica* was observed in the back and lateral areas of the tongue and saliva (15,16). *P. melaninogenica* has hemagglutinating activity and agglutinates red blood cells despite its low pathogenicity (17). It has been observed that oral bacteria that survive the acidic conditions of the stomach gain access to the small intestine and large intestine, where they can interact with intestinal bacteria. Also, some studies recommended that *P. melaninogenica* was significantly increased in both ulcerative colitis and Crohn's disease patients, thus suggesting *Prevotella* as a potential predictor for these disorders (18).

This study aimed to examine *P. melaninogenica* in a group of UC patients and compare it with a control group to ascertain whether the oral cavity also demonstrates bacterial dysbiosis in the Golestan province, northeast of Iran.

Methods

This case-control study was conducted on patients with ulcerative colitis who were referred to the gastroenterology department of Shahid Sayad Shirazi Hospital in Gorgan between 2019 and 2020. Eighty individuals, including 40 UC patients and 40 healthy controls (HCs), participated in the study. A gastroenterologist conducted a clinical examination of the participants using the disease activity index. Individuals were identified as patients with IBD based on the criteria of history, clinical symptoms, clinical images, histopathology, and clinical standards. Healthy controls who did not have any known systemic diseases were selected after matching with the UC patients in terms of age and gender. The exclusion criteria were using systemic and topical antibacterial agents (such as chlorhexidine) in the last month, smoking in the last five years, and using corticosteroids in the last two years.

The study protocol was approved by the Ethics Committee of the Golestan University of Medical Science (IR.GOUMS.REC.1398.138) and was carried out following the rules outlined in the Helsinki Declaration. Before the study, all the participants were provided with detailed information about the study. After understanding the details, they signed a written consent form to confirm their willingness to participate.

The saliva samples were collected for both groups. Subjects were required to refrain from eating or drinking for one hour before taking the saliva samples. Saliva stimulation was obtained from individuals by directly spitting in two sterile plastic tubes. The samples were kept at -80 degrees Celsius until processing.

The DNA was extracted from a saliva sample using a DNA extraction kit (Favorgene). To extract DNA from saliva samples, suspensions were created in 400 microliters of buffer (TES (10 mM Tris [pH 8]), 5 mM EDTA, 50 mM NaCl) and centrifuged at 2000 rpm for 5 minutes to separate large particles. The supernatant was centrifuged again at 7000 rpm for 5 minutes to sediment the bacteria, and then DNA extraction was performed according to the kit protocol. At 260 nanometers, the isolated DNA's optical density (OD) was calculated. Then, the DNA was kept at minus 20 degrees (−20 °C) for real-time PCR.

The 16S rRNA gene sequence of *P. melaninogenica* was identified in the GenBank database, and the sequences were then aligned. The desired primer was finally verified (19) (Table 1). Each real-time PCR was performed in a 25-l volume with 10–100 picograms of template DNA and 100 nanomolar of each forward and reverse primer. The real-time PCR settings were 40 cycles of 95 °C for 15 s and 60 °C for 1 min each, followed by 50 °C for 2 min and 95 °C for 10 min (19).

Table 1. Sequences of oligonucleotide primers of P. *melaninogenica* (19)

Primers and probe	Sequence (5′ → 3′)	Tm (°C)
Forward	CCAGCCAAGTAGCGTGCA	58.1
Reverse	TGGACCTTCCGTATTACCGC	58.5

Quantitative traits were calculated by estimating the mean and standard deviation, and frequency distribution tables were used for qualitative or classified data. According to the study design and the data features, if the normality of the data distribution was confirmed based on the Shapiro-Wilk test and the homogeneity of the variances based on the Lone test, the independent t-test was used to compare the average data in two groups. If the homogeneity of variances was not confirmed, the Welch t-test was applied. If the normality of the data distribution was not confirmed, the Mann-Whitney test was used. The analysis of the covariance method was used to adjust quantitative confounding variables, and the Mantel-Haenszel method for categorical variables was devoted. All data were analyzed using SPSS version 22.

Results

According to the independent t-test, the average age of people in the two groups was not significantly different (p =0.170). In addition, based on the chi-square test, the gender distribution in the two groups was not statistically significant (p =0.215).

As shown in Table 2, the frequency of *P. melaninogenica* in UC patients (77.5%) was significantly higher than in the control group (45%) (p = 0.003). The copy number of *P. melaninogenica* in UC patients (339 ± 1082.29) was also significantly higher than in the control group (61.29 ± 154.03) (p = 0.005). In women with UC, the number of *P. melaninogenica* was significantly higher than in the control group (p = 0.0342). Similarly, in men, the UC group (262.85 ± 664.97) had a significantly higher number than the control group (72.62 ± 222.76) (p = 0.015) (Table 2).

Table 2. Demographic characteristics, the frequency and copy number of *P. melaninogenica* in UC patients, controls, and its distribution among females and males

Variable		UC n=40	Control n= 40	*p*-Value
Age (Mean±SD)		41.24±9.9	44.55±10.52	0.170
Gender n (%)	Female	20 (50%)	29 (72.5%)	0.215
	Male	20 (50%)	11 (27.5%)	
***Prevotella melaninogenica*, n (%)**		Negative: 9 (22.5%), Positive: 31 (77.5%)	Negative: 22 (55%), Positive: 18 (45%)	0.003
***P. melaninogenica* (Mean±SD)**		339.31±1082.29	61.29±154.03	0.005
***P. melaninogenica* in males (Mean±SD)**		262.85±664.97	72.62±222.76	0.015
***P. melaninogenica* in females (Mean±SD)**		492.35±1427.61	56.98± 123.50	0.0342

Discussion

Ulcerative colitis is a chronic and relapsing inflammatory disease of the large intestine that leads to the destruction and inflammation of the colonic mucosa. The main cause of this disease has not been discovered yet. However, it has been observed that some bacteria may play a role in predisposing patients to this disease. Previous studies have investigated the role of gut bacteria in UC. However, microbiota dysbiosis in other tissues, including the oral mucosa, has received less attention. *Streptococcus*, *Prevotella*, *Haemophilus*, and *Veillonella* are bacteria that have been extensively researched in this context. The presence of *Prevotella* may affect, positively or negatively, the severity of diseases, such as gastritis, Crohn's disease, UC, and cancers. *Prevotella* spp. are obligate anaerobes that are isolated from the mouth cavity and primarily found on mucosal surfaces (20). *Prevotella* is thought to be a beneficial gut species since it is seen in greater abundance in the gut when a high-carbohydrate diet is followed (21). However, *Prevotella* species and other members of the natural microflora are responsible for a number of local reactions, including UC, arthritic conditions, and gastrointestinal disorders (22). Dysbiosis changes the immune system, disrupts homeostasis, and increases the production of several virulence factors; in this regard, it can cause *Prevotella* species to turn from a normal bacterium into a pathobiont. With the help of these factors, they can survive regardless of environmental change. Thus, they survive and inhibit the infected area. The interplay between pathogenicity and the host's defense mechanisms against them determines the onset and course of the disease (23,24).

In this regard, this study aimed to investigate the frequency of *P. melaninogenica* bacteria in UC patients compared to the control subjects, and the intensity of this bacterium was investigated in two groups. According to the results, the frequency of *P. melaninogenica* in UC patients was higher than in the healthy controls, and this difference was statistically significant (p=0.003). In a study conducted by Qi et al., *Prevotella* (besides *Veillonella*) and *P. melaninogenica* significantly increased in both Crohn's disease and ulcerative colitis patients. The genus *Prevotella* was proposed as a potential marker linked to IBD, especially Crohn's disease (18). In addition, a 2017 study revealed greater levels of several oral bacteria in the feces microbiota of UC patients, identifying species from the genus *Prevotella* (25). In another study, it was shown that the dysbiosis of distinct salivary microbiota was influential in the development of IBD. Some bacteria, including *Leptotrichia*, *Prevotella*, *Bolidia*, and *Atopobium*, showed a significant increase (18). However, in a study conducted in 2022, different species of *Streptococcus*, *Uribacterium*, *Ruthia*, *Provetella*, and *Porphyromonas* were not found in the saliva samples of the UC group (26). In this regard, it is noteworthy that the saliva samples of UC patients lacked several species that are important components of the oral core microbiome, such as *Prevotella* members (*P. salivae*, *P. histicola*, and *P. melaninogenica*). This finding has also been observed in IBD patients (27).

In addition, results showed a significantly higher count of *P. melaninogenica* bacteria in the patient group compared to the control group. A study by Ying Qi et al. showed that the dysbiosis of distinct salivary microbiota, such as *Prevotella*, which increased significantly, is influential in the development of IBD (18).

Conclusion

In conclusion, this study described that higher frequency and number of P. *melaninogenica* in UC patients' saliva could contribute to the oral dysbiosis observed in UC. This data supports the hypothesis that UC may be associated not only with gut microbial imbalance but also with other microbial community imbalances, such as oral dysbiosis. Currently, there is limited data to describe the relationship between *P. melaninogenica* in the oral cavity and gastrointestinal disorders. Further investigation on a larger scale with a more powerful design is necessary to recommend this bacterium as an oral biomarker for UC.

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Ethical statement

This study received ethical approval from the Golestan University of Medical Sciences Ethical Committee in Iran (IR.GOUMS.REC.1398.138). The study was conducted in accordance with the current and seventh edition of the Declaration of Helsinki. All participants provided written informed consent.

Conflicts of interest

The authors declare no conflict of interest.

Author contributions

SZ assisted in conceptualization, methodology, supervision, original draft preparation, review, and editing. SB assisted in conceptualization, review, and editing. NB assisted in statistical analysis, review, and editing. NA assisted in data collection and original draft preparation. AB assisted in sample collection. NM assisted in conceptualization, methodology, supervision, original draft preparation, review, and editing. All authors have read and approved the final manuscript.

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