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Isolation and Identification of Bacteria Associated with Irritable Bowel Syndrome

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Abstract

Background: Irritable bowel syndrome (IBS) represents a multifactorial functional gastrointestinal disorder which affects approximately 10-15% of the world's population. It has been demonstrated that microbial disturbances are a crucial part of its etiology and sustainment. Unfortunately, a lack of complete bacteriological profiling of stool specimens of patients from some geographic locations exists. **Objective:** The objective of the work is isolation and identification of bacterial species from stool specimens of patients diagnosed with irritable bowel syndrome and the comparison of their profiles with those of healthy control subjects as well as the determination of the resistance of the isolated strains to various antimicrobial drugs. **Methods:** Stool specimens were taken from 80 patients who met the Rome IV Irritable bowel syndrome diagnostic criteria and 40 healthy age- and sex-matched control subjects. Traditional bacteriological cultures were followed by sequencing of the *16S RNA* gene in order to definitively establish the species of isolates. Antibiotic susceptibilities were measured according to CLSI criteria via the Kirby-Bauer disk diffusion test. **Results:** In total, 248 bacterial isolates have been identified in stool specimens of IBS patients, while only 98 isolates have been found in controls. Among isolates obtained from IBS patients, the most frequent included *E. coli* (31.5%), *K. pneumoniae* (18.1%), *E. faecalis* (14.1%), *B. fragilis* (11.3%), *lactobacilli* (9.3%), *bifidobacteria* (7.7%), along with other lesser represented bacterial strains A decrease in the number that is statistically significant of *lactobacilli* and *bifidobacteria* ($p<0.01$) was found in IBS patients. Regarding antibiotic resistance, Enterobacteriaceae showed significant resistance to ampicillin (58.4%) and tetracycline (47.6%).

Conclusions: It has been found that IBS patients have significant dysbiosis in terms of reduced numbers of beneficial bacteria with concomitant colonization by pathogenic bacteria.

Keywords: Irritable bowel syndrome; gut microbiota; dysbiosis; bacterial isolation; 16S rRNA; antimicrobial resistance; Rome IV criteria.

Introduction

Irritable bowel syndrome (IBS) is a prevalent gastrointestinal condition, impacting 10-15% of adults globally, with distribution varying by geographical region and diagnostic criteria employed. The condition is characterised by recurring stomach pain and discomfort, accompanied by constipation and/or diarrhoea, without any observable morphological or chemical abnormalities [1]. It inflicts significant suffering on patients, negatively impacts their quality of life, and imposes a substantial strain on healthcare systems. The pathophysiology of IBS is acknowledged to involve multiple elements, including as modifications in the gut-brain axis, gastrointestinal hypersensitivity, motility abnormalities, inflammatory alterations in the gut mucosa, and immune system activation [2]. Recent scientific study has demonstrated that the composition and functionality of the gut microbiota play a crucial role in the onset and maintenance of IBS symptoms.The human gut has a complex microbial community primarily composed of bacteria, whose metabolism regulates different physiological and neurological processes occurring within it.[3] Changes in composition, known as dysbiosis, of the gut microbiota have been identified as a characteristic feature of IBS in multiple investigations. A reduced presence of advantageous commensals from the genera Lactobacillus and Bifidobacterium, alongside an elevated proportion of bacteria associated with inflammatory responses, including *E. coli*, *Klebsiella*, and some Bacteroides species, has been noted.[4] A potential factor contributing to the development of IBS in certain people is the presence of small intestine bacterial overgrowth (SIBO). A subset of IBS-D individuals has been identified with lactulose breath tests revealing elevated hydrogen production, indicating bacterial colonisation of the small intestine. Post-infectious IBS, which arises following acute gastrointestinal infections caused by pathogens such as *C. jejuni*, *Salmonella spp*., and *Shigella spp*., is a recognised condition that underscores the etiological significance of bacteria [5]. Given the aforementioned, it is evident that a comprehensive characterisation of isolated bacteria by traditional culture methods combined with molecular analysis is essential for microbiological monitoring. Indeed, this will facilitate the recovery of viable bacteria that may undergo antibiotic susceptibility testing, yielding critical clinical information [6]. This study was meticulously crafted in accordance with these principles to guarantee clinical uniformity. The study aimed to culture bacteria from faecal samples of IBS patients, as classified by Rome IV criteria; compare their profiles with those of a healthy control group; assess the sensitivity of the isolated microorganisms to various antibiotics; and furnish supplementary bacteriological data regarding the role of gut microbiota in IBS.

Materials and Methods

Study Participants

A total of 80 patients diagnosed with IBS according to the Rome IV criteria attended the Gastroenterology Outpatient Department clinic. Individuals with a four-week history of antibiotic use before to sample collection, inflammatory bowel illnesses, colorectal malignancy, coeliac disease, and any other organic gastrointestinal pathology were excluded from the study population. Furthermore, forty age- and gender-matched healthy persons devoid of gastrointestinal symptoms and who had not ingested any antibiotics in the four weeks preceding sample collection were designated as the control group.

Sample Size Calculation. The sample size estimation was conducted a priori utilising G*Power software (version 3.1; Faul et al., 2007). A two-proportion z-test was used based on the anticipated dysbiotic bacteria proportions of approximately 65% in IBS patients and 30% in healthy controls, as outlined in a comparable prior study (Tap et al., 2017). With a two-sided $\alpha = 0.05$, statistical power (1-β) of 0.80, and an effect size $h = 0.72$ (Cohen's h), a minimum of 38 participants per group was established. Alongside the sample size calculation, accounting for an anticipated attrition rate of around 10% and statistical precision, 80 IBS patients and 40 healthy controls were recruited, yielding a case-to-control ratio of 2:1. A 2:1 ratio was employed to enhance statistical efficiency while maintaining recruitment feasibility in the clinical environment.

Sample Collection and Transportation

Fresh stool samples were collected from subjects and stored in sterile, leak-proof, and screw-capped tubes. Samples were transferred to the Microbiology laboratory within two hours of their collection and analyzed immediately. Otherwise, stool samples were refrigerated at 4°C and processed within four hours post collection, if necessary.

Bacteriological Cultivation

All the obtained stool samples were cultured using selective and non-selective culture media, such as BA, MCA, MSA, MRS agar (used for isolation of lactobacilli), BSA, and EMB agar. All the plates were incubated aerobically for 24-48 h at 37°C. Plates MRS and BSA were incubated anaerobically for 48-72 h at 37°C. Colony appearance, color, presence of hemolysis, and Gram stain were characterized for each morphological isolate.

***16S rRNA* Gene Sequencing for Identification of Bacteria**

Species-level identification was accomplished via *16S rRNA* sequencing in instances where isolates exhibited ambiguous morphological or biochemical characteristics. Genomic DNA was extracted from pure cultures using the QIAamp DNA Mini Kit (QIAGEN, Germany) following the manufacturer's guidelines. . The *16S rRNA* gene was amplified utilising universal primers, 27F (5′-AGAGTTTGATCMTGGCTCAG-3′) and 1492R (5′-TACGGYTACCTTGTTACGACTT-3′). A segment of 1465 base pairs in length, encompassing all nine hypervariable regions (V1-V9) of the *16S rRNA* gene, was amplified utilising a 50 µL reaction mixture composed of 25 µL 2x KAPA HiFi HotStart ReadyMix, 1 µL of each primer (10 pmol/µL), 2 µL of template genomic DNA, and nuclease-free water to achieve a final volume of 50 µL. The reaction conditions included a denaturation phase of 3 minutes at 95°C, followed by 35 cycles consisting of 30 seconds at 95°C, 30 seconds at 55°C, and 90 seconds at 72°C, concluding with an extra elongation of 10 minutes at 72°C. Amplicons were analysed on a 1.5% agarose gel stained with ethidium bromide and examined under UV light.

Antibiotic Susceptibility Test

Antibiotic susceptibility test of significant isolates was done using the Kirby-Bauer disk diffusion technique on Mueller-Hinton agar in accordance with CLSI standards. The following antibiotics were used: ampicillin (10 µg), ciprofloxacin (5 µg), amoxicillin-clavulanate (20/10 µg), trimethoprim-sulfamethoxazole (1.25/23.75 µg), tetracycline (30 µg), ceftriaxone (30 µg), gentamicin (10 µg), imipenem (10 µg), and metronidazole (5 µg). Interpretations of the susceptibility tests results were performed according to CLSI criteria. *Escherichia coli* ATCC 25922 was used as the quality control strain.

Statistical Analysis

Data were inputted and analysed utilising SPSS Statistics version 26.0 (IBM Corp., Armonk, NY, USA) and R version 4.3.1 (R Core Team, 2023). Descriptive statistics were employed to summarise bacterial isolation frequencies and demographic data, presented as frequencies and percentages for categorical variables, and as means ± standard deviations (SD) or medians with interquartile ranges (IQR) for continuous variables, contingent upon data distribution as assessed by the Shapiro-Wilk normality test. A two-tailed p-value of ≤0.05 was deemed statistically significant for all analyses.

Results

Demographic Characteristics

Of the 80 patients included in this study suffering from IBS, 47 (58.8%) were females, whereas 33 (41.3%) were males with an average age of 34.6 ± 11.2 years (age range 18-62 years). Subtypes of IBS were distributed as follows: 34 (42.5%) for IBS-D, 26 (32.5%) for IBS-C, and 20 (25.0%) for mixed-type IBS (IBS-M). The control group consisted of 40 healthy subjects whose average age was 33.9 ± 10.8 years. Age and sex differences between the two groups were not statistically significant. ($p > 0.05$). A total of 248 bacterial strains from 12 different types of bacteria were isolated from stool samples of IBS patients with an average of 3.1 isolations per stool sample. On the other hand, a total of 98 bacterial strains were isolated from the stool samples of healthy subjects with an average of 2.45 isolations per sample. The total number of bacterial strains isolated in both groups differed statistically significantly ($p < 0.001$). The distribution of bacterial strains in both groups is summarized in Table 1.

Table 1. Distribution of bacterial isolates from IBS patients and healthy controls

Bacterial Species	IBS Patients (n)	IBS %	Controls (n)	Controls %
Escherichia coli	78	31.5	20	20.4
Klebsiella pneumoniae	45	18.1	9	9.2
Enterococcus faecalis	35	14.1	8	8.2
Bacteroides fragilis	28	11.3	6	6.1
Lactobacillus spp.	23	9.3	28	28.6
Bifidobacterium spp.	19	7.7	18	18.4
Clostridium perfringens	10	4.0	3	3.1
Staphylococcus aureus	5	2.0	2	2.0
Proteus mirabilis	3	1.2	1	1.0
Other species	2	0.8	3	3.1
Total	**248**	**100.0**	**98**	**100.0**

Antimicrobial Susceptibility

Among Enterobacteriaceae isolates, resistance to ampicillin was the most common phenotype, detected in 58.4% of isolates, followed by tetracycline (47.6%), trimethoprim-sulfamethoxazole (39.2%), and ciprofloxacin (22.8%). All Enterobacteriaceae isolates remained sensitive to imipenem. E. faecalis isolates demonstrated high susceptibility to ampicillin (91.4%) but resistance to tetracycline (62.9%) and trimethoprim-sulfamethoxazole (45.7%). B. fragilis isolates showed high susceptibility to metronidazole (96.4%) and imipenem (100%). Table 2 presents the complete antimicrobial resistance profiles.

Table 2. Antimicrobial resistance rates (%) among selected bacterial isolates from IBS patients

Antimicrobial Agent	E. coli %R	K. pneumoniae %R	E. faecalis %R	B. fragilis %R
Ampicillin	61.5	55.6	8.6	N/A
Ciprofloxacin	24.4	20.0	17.1	N/A
Tetracycline	50.0	42.2	62.9	N/A
TMP-SMX	41.0	37.8	45.7	N/A
Ceftriaxone	19.2	22.2	N/A	N/A
Gentamicin	12.8	15.6	20.0	N/A
Imipenem	0.0	0.0	N/A	0.0
Metronidazole	N/A	N/A	N/A	3.6

TMP-SMX = Trimethoprim-Sulfamethoxazole; %R = Percentage Resistant; N/A = Not applicable for this species.

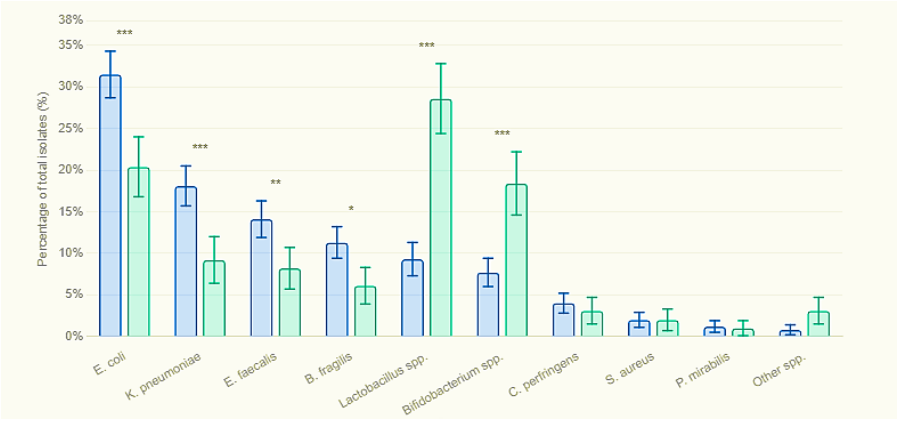

Figure 1. *Comparative distribution of bacterial isolates in IBS patients and healthy controls.* Grouped bar chart illustrating the percentage frequency of each bacterial species recovered from stool specimens of IBS patients.

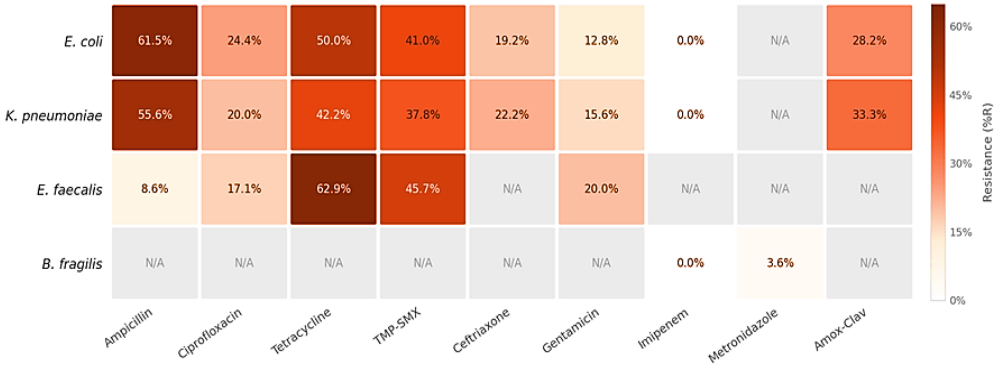

Figure 2. *Antimicrobial resistance heatmap of selected bacterial isolates from IBS patients.* Heatmap depicting the percentage resistance (%R) of four predominant bacterial species (*E. coli*, *K. pneumoniae*, *E. faecalis*, *B. fragilis*).

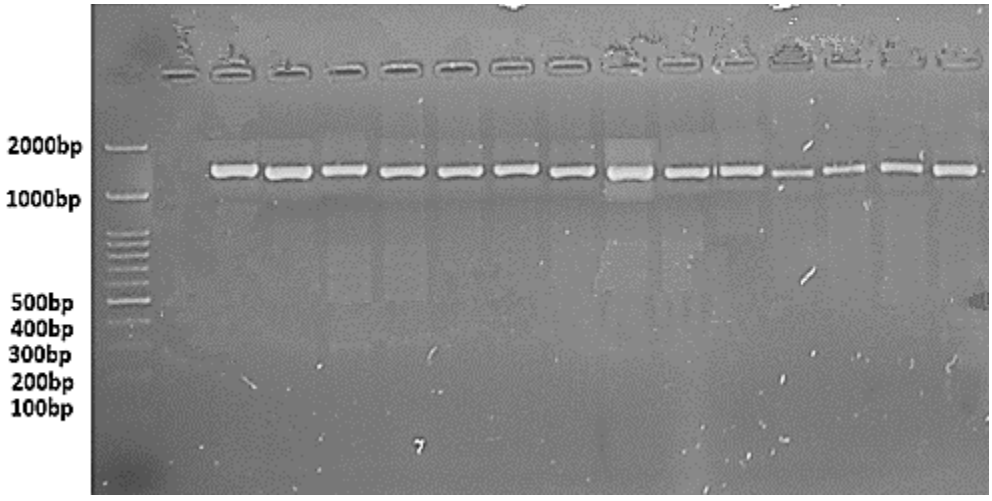

Fig 3. The *16S rRNA* gene was amplified by polymerase chain reaction (PCR) using universal primers at 1465 bp region spanning the full-length *16S rRNA* gene.

Discussion

This study provides detailed bacteriological profiling of gut microbial signatures in individuals diagnosed with IBS in accordance with Rome IV guidelines and identifies a signature of marked dysbiosis involving significant changes in both bacterial load and community composition. Increased levels of total bacteria were noted in IBS patients compared with healthy subjects, a trend previously described in various culture-based and molecular epidemiological studies. [7] Preponderance of *E. coli* in the isolates recovered from IBS patients correlates with existing data suggesting a causal relationship between these bacteria and IBS development. Some pathotypes of *E. coli*, including adherent invasive E. coli, exhibit the capacity for mucosal colonization, induction of proinflammatory cytokines, and disruption of epithelial barrier in susceptible individuals.[8] Similarly, higher prevalence of *K. pneumoniae* found in our study cohort is of interest, considering the involvement of these organisms in experimentally induced gut inflammation and immune response imbalance.[5] The decreased numbers of *lactobacilli* and *bifidobacteria* observed in IBS patients relative to control individuals represents one of the most frequently reported aspects of dysbiosis. These bacteria contribute to gut health by means of mucosal protection via short-chain fatty acid production, competition for colonization niches with pathogens, and interaction with the immune system both innate and adaptive.[9] Therefore, the observed alteration of gut flora may serve as an important mechanism of pathogenesis, justifying probiotic administration in IBS management.[6] The association of B. fragilis with IBS-C observed in this study corresponds with the existing knowledge of enterotoxigenic strains of this organism contributing to motility disturbances, and hence, to the formation of constipation dominant IBS. The existence of subtype-specific microbial patterns further emphasizes the heterogeneity of this syndrome and supports the idea of distinct mechanisms responsible for IBS manifestation.[9] High prevalence of ampicillin and tetracycline resistance among the isolates of Enterobacteriaceae in this study corresponds with trends reported on a global level, reflecting the development of resistance to antimicrobials in the commensal flora.[10] The clinical importance of this finding can be explained by the possibility of selective enrichment of resistant strains in the course of inappropriate antibiotic therapy. Importantly, some antibiotics, such as non-systemic neomycin and rifaximin, used for SIBO associated IBS management, are not expected to contribute to this problem. [11] Limitations include a cross-sectional study design, which does not allow evaluation of the temporal sequence of events involved in IBS development. Moreover, despite clinical importance, culture-based methods employed in this study fail to account for the number of anaerobic and fastidious microorganisms representing an important part of normal gut flora; therefore, future studies would benefit from metagenomic analyses.[12] It should be noted that the diet of the participants was not considered in the current study as another important determinant of microbial diversity and constitutes a source of potential bias.[7] Despite the aforementioned shortcomings, the results obtained provide valuable bacteriology information about the population understudied previously. They confirm the hypothesis of gut dysbiosis being a crucial aspect of IBS and justify the consideration of targeted interventions aimed at the restoration of microbiota balance using probiotics, prebiotics, dietary modifications, and appropriate use of antibiotics as promising directions of research. [9]

Conclusion

In conclusion, the results indicate that IBS is associated with quantitative and qualitative changes in the fecal microbiota, favoring potentially harmful bacteria over beneficial ones, alongside elevated antimicrobial resistance. These findings reinforce the importance of microbiota-targeted therapies (probiotics, prebiotics, or dietary interventions) in IBS management. Larger, multicenter studies incorporating next-generation sequencing and functional metagenomics are recommended to further elucidate causal relationships and identify specific microbial biomarkers for personalized IBS treatment.

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