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RESEARCH ARTICLE

Molecular Diagnosis of Bacteria Isolated from Colorectal Cancer Patients with Potential Use as A Diagnostic Tool for The Disease

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ABSTRACT

Background: The human gut environment is colonized by a vast and diverse microbial community, vastly outnumbering the cells of the human body itself, many of which remain unidentified Microorganisms inhabit nearly all areas of the human body, possessing a diverse array of genes. These genes can interact with, alter, or deactivate numerous human genes, particularly within colon cells. "..

Aim of study: Isolation and molecular characterization of intestinal bacteria associated with colorectal cancer and their role in tumorigenesis.

Methods:137 different samples were collected from infected individuals. The samples included blood, stool, and biopsy taken from the endoscopy unit inside Al-Sadr Teaching Hospital in Basra Governorate during the period from March 2022 to December 2023.

Results: The bacteria associated with colorectal cancer patients were isolated and molecularly characterized by 16S rRNA sequencing. 27 different species were identified, including enteric genera, rare genera, and anaerobic genera.

Conclusion: The study identified bacteria such as Fusobacterium nucleatum, E. coli, Salmonella enterica and other species associated with the disease. These bacteria contribute to the development of the tumor and can serve as diagnostic markers for its detection.

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INTRODUCTION

The human gut mucosa is a complex ecosystem comprised of epithelial cells, lamina propria, and muscularis mucosae. This environment is colonized by a vast and diverse microbial community, numbering approximately 1014 microorganisms, which significantly outnumbers the human body's own cells. These commensal bacteria, predominantly anaerobic strains, establish residence in the intestinal tract immediately postpartum and encompass estimated A thousand species, many of this remain unidentified.(Zhang et al.,2015). Microorganisms inhabit nearly all regions of the human body, possessing a vast number of genes. These microbial genes can interact with, modify, or disrupt a wide range of human genes, particularly within colon cells. Notably, the human microbiome encodes for approximately 100 times more proteins than the human genome itself. This diverse

microbial community encompasses 1000 to 1500 bacterial species, and its composition varies significantly between individuals (Serino , 2018) . Gut bacteria not only contribute to the host's gut defense system but also play a crucial role in maintaining normal gut function. These beneficial effects encompass a wide range of activities, including regulating gut motility, synthesizing vitamins, transforming bile acids and steroids, metabolizing foreign substances, facilitating mineral absorption, and activating or deactivating toxins, genotoxins, and mutagens (Macfarlane et al.,2009) Microorganisms inhabit nearly all regions of the human body, possessing a vast number of genes. These microbial genes can interact with, modify, or disrupt a wide range of human genes, particularly within colon cells. Notably, the human microbiome encodes for approximately 100 times more proteins than the human genome itself. This diverse microbial community encompasses 1000 to 1500 bacterial species, and its composition varies significantly between individuals (Serino , 2018) The gut microbiome primarily comprises bacteria from a few major phyla, including Firmicutes, Bacteroidetes (the most abundant), Proteobacteria, Verrucomicrobia, Actinobacteria, Fusobacteria, and Cyanobacteria. While the overall distribution of these bacterial types and their abundance is generally consistent in healthy individuals, significant variations occur in individuals with diseases. Recent research has revealed the presence of eukaryotic fungal species alongside bacteria within the gut microbiome, although bacteria remain the predominant component (Sommer and Bäckhed , 2013; Górska et al.,2018) It is well-established that gut microbiota significantly influence the development of colorectal cancer (CRC), either through their metabolites or by interacting with intestinal epithelial cells. An imbalance in this microbial community has been linked to various disorders, including inflammatory bowel disease and CRC. While some studies suggest that alterations in the microbiota contribute to the onset of disease, others indicate that these changes may be a consequence of the disease process. Further research is crucial to definitively determine the causal relationship between microbial dysbiosis and CRC (Harris et al., 2016; Wang et al., 2018). Colorectal cancer arises from the accumulation of genetic mutations. Extensive research has identified approximately 13,000 mutations across 67 genes associated with CRC, although only 12 of these genes have been definitively linked to the disease. Genomic instability, particularly chromosomal instability (CIN), plays a crucial role in CRC development. CIN, characterized by gains or losses of entire chromosomes or chromosomal segments, is implicated in approximately 85% of CRC cases. Common chromosomal alterations in CIN-driven CRC include the loss of segments on chromosomes 15q11-q21, 17p12-13, and 18q12-21, and the gain of segments on chromosomes 1q32, 7p, 7q, 8q, 13q, 20p, and 20q. (Sabit et al., 2019).

The observed differences in gut microbiota composition between individuals with colon adenomas or colorectal cancer and healthy individuals suggest that specific alterations in fecal microbiota and their metabolites may serve as promising biomarkers for early CRC screening (Zhao et al., 2021). Fecal bacteria have been further investigated as a potential non-invasive biomarker for colorectal cancer detection. In a group of patients with colorectal cancer and a group of healthy subjects, the presence of F. nucleatum in stool samples from cancer patients was identified as a potential biomarker for the onset of colorectal cancer. The presence of F. nucleatum alone showed a high sensitivity and specificity of approximately 80%. (Liang et al., 2017; Eklöf et al., 2017) . Just as the combination of a number of types of bacteria in the stool, such as F. nucleatum, Bacteroides clarus, and Clostridium hathewayi, led to an increase in the detection rate, the combination of these bacteria with the result of the fecal immunochemical test increased the sensitivity of colorectal cancer detection to 92.8% and even 100% in the early stage of colorectal cancer detection.(Montalban-Arques et al., 2019) Given its established ability to damage genetic material., Colibactin has emerged as a potential non-invasive biomarker for colorectal cancer (CRC), detectable through readily accessible samples such as stool or rectal swabs. Numerous studies have investigated the prevalence of colibactin in CRC patient samples compared to healthy controls to assess its diagnostic utility. For instance, the clbA gene, associated with colibactin production, was detected using qPCR in stool samples from individuals referred for colonoscopy due to lower gastrointestinal symptoms.

MATERIAL AND METHODS

Study of group

The study was conducted on suffering from problems in the digestive system, specifically in the intestines and rectum, one hundred thirty seven samples were collected from patients with different infections including samples were divided into two groups, aerobic and anaerobic, Aerobic blood (n=30) anaerobic blood (n=28), biopsy (n=20), rectal swab including samples were divided into two groups , rectal swab aerobic (n=30) and rectal swab anaerobic (n=29) during the period from march 2022 to December 2023., where a group of The patients went to the endoscopy unit at Al-Sadr Teaching Hospital in Basra, where they were diagnosed with colorectal cancer through colonoscopy examination and under the supervision of the specialist doctor, and another group went to the Oncology and Hematology Center in Basra, and a group went to the Basra Center for Radiotherapy, where the last two groups were diagnosed with the tumor in The patient beforehand, and the study included the collection of three different types of samples, namely a biopsy from the area of tumor and blood, in addition to the stool.

Show all of these individuals Signs and symptoms including Changes in bowel movements, Cramps and bloating Blood in the stool, abdominal pain, unexplained weight loss, fatigue Shortness of breath (Holtedahl et al, 2021). Direct interviews were used with each patient completing a questionnaire that included: name, age, gender, address and smoking. It covers questions related

to epidemiological and clinical features and laboratory investigations of patients.

Inclusion criteria:

Patients with colorectal cancer who meet the diagnostic criteria and were diagnosed as colorectal cancer

Exclusion criteria:

• Those who currently have intestinal infection.
• Those who had used antibiotics or microecological agents within 2 months before enrollment.
• Patients who received adjuvant chemoradiotherapy before sampling.
• Those who suffer from chronic diseases such as hypertension, heart disease, and diabetes.
• Those who refuse to participate in our study.

Ethical Approval :

The necessary ethical approval was obtained by verbal consent from patients. This study was approved by the committee of publication ethics at Training and Development Center\Basra Health Department from number 16/2021

5 Specimens Collection and Processing (culture).

Preparation of culture media

All cultures media including Schaedler anaerobe agar, Thioglycollate broth Brain heart infusion broth(B.H.I broth) XLD agar (Xylose Lysine Deoxycholate), MacConkey Agar, SS agar (Salmonella-Shigella agar) Blood base agar, Nutrient agar, Nutrient broth and peptone broth were prepared according to the instructions of their companies and autoclaved at 121°C for 20 min Except for SS agar and XLD agar It is not processed in autoclave or overheat , then dispensed into sterile Petri dishes or tubes as required and incubated for 24 hours at 37°C to ensure sterility, and stored at 4°C until use.

blood specimens

The cover of the aerobic BACTEC bottle was removed, and then the site was disinfected using 70% alcohol for a period of not less than 30 seconds using a circular motion and allowed to dry completely in the air for 1 minute. Then, using a syringe, 10 ml of the patient's blood was withdrawn and placed in a BACTEC bottle, and then the sample was mixed. Well and incubated for 7 days in the BD Bactec device, where the changes are noticed. Enter the tube during the incubation period by producing CO2, as it is evidence of the growth of pathological bacteria, after which the sample is cultivated on a blood plate.

stool specimens for culture.

Stool and rectal-swab samples are collected from patient Through a swab with Media, where the sample was collected through the anus, and it must be confirmed that it contains stool, placed in the swab, and then transported to the laboratory by means of a cool box.

Biopsy Specimens

Two biopsies are taken from the tumor area using a colonoscopy, and the biopsies are placed in a screw cap tube filled with Thioglycollate broth to a volume of 25 ml. It is closed tightly to ensure that no air enters the mechanism and is placed in a cool box until it reaches the laboratory.after all collected samples transferred to bacteriology laboratory within 2 hrs and incubated at 37 °C for 24-48 hrs.Where about 50 microliters of the sample was taken by inserting a micropipette into the bottom of the Screw cap tube, where inoculated on schaedler agar and Blood base agar, and a layout was made on Petri dishes. Then the dishes are placed in an anaerobic environment.

Isolation of bacterial

The positive samples for bacterial growth were sub-cultured to get a pure colony, then cultured again on , The bacterial isolate was inoculated into BHIB broth and incubated at 37ºC for 24 hours then the broth culture was preserved by adding glycerol to a final concentration of 20% and stored at -20ºC for 12-18 months.

Identification of Escherichia coli bacteria

Morphology of the bacteria Colony

Morphology of the bacterial Colony: Bacterial colony morphology was determined by observing colony characteristics on MacConkey Agar, Blood Agar, and Nutrient Agar, followed by Gram staining and microscopic examination (Farhan and Al-iedani, 2021; Tharia et al., 2024).

The genomic DNA extraction and Bacteriological identification with16S rRNA:

DNA has been extracted according to (Favorgen Kit). As the procedure was elucidated in the user´s manual the genome of the E.coli isolates was identified by using PCR to amplify universal bacterial 16S rRNA primers For bacteria E.coli 27F 5'-AGAGTTTGATCCTGGCTCAG-3' and 1492 R 5'-GGTTACCTTGTTACGACTT-3' (Miyoshi et al., 2005). total volume of 25 µl were used for PCR amplification, F Primer 1 µl, R Primer 1 µl, DNA template 3 µl, master mix 12.5µl, Band docter 2.5µl and Nuclease-free water 5 µl were added in the Master Mix tube 25 µl. where The Initial denaturation temperature was 95˚C for 5 min with 1 cycle , 30 cycle of denaturation 95˚C for 30 Sec, Annealing 55.˚C for 30 Sec, Extension 72˚C for 1 min . The Final extension was carried out at 72˚C for 5 min with 1 cycle . three µl of PCR product was loaded inside the well and usually 3 µl of 1000 bp DNA ladder was loaded inside the first well for comparing the size of the amplified gene . The running was for 1 h at 70 volt then the bands were detected under UV transilluminator and photographed.

Sequencing of the 16S rRNA

Preparation of Samples: The unpurified PCR products of 16S rRNA gene which will be sent for purifying and perform the. each tube of samples was branded with numbers which are matching to those of Excel sheets which 20µl of PCR product for 16S rRNA was sent to to Macrogen in koria .

Analysis of Gene Sequencing Data: Being treated and corrected, sequence products are copied and pasted in the box of "BLAST "which has been loaded from website http://blast.ncbi.nlm.nih.gov 46. bacteria, names will immediately appear accompanied with percentages of sequence compared with gene bank data.

RESULTS

Isolation of bacteria

The 86 bacterial isolates were obtained from 137 (62.7 %) Samples from diverse origins Table1. There were 59 of 59 (100%) from rectal swap , 58(25.8%) from blood , 20 (60%) from biopsy, , isolates with significant differences in comparison to other sources at $P \leq 0.05$.

Table : 1 The number of samples and isolates according to their sources.

Table : 1 The number of samples and isolates according to their sources

Sample sources	*no. of samples*	*no. of Isolates n (100%)*
Rectal swap	*59*	*59 (100)*
Blood	*58*	*15 (25.8)*
Biopsy	*20*	*12 (60)*
Total	*137*	*86(62.7)*

Dentification of bacterial species by 16S rRNA gene

The 16S rRNA of 86 bacterial isolates was shown on agarose gel electrophoresis and its position was approximately 1500 bp comparing with a molecular DNA ladder Fig : 1

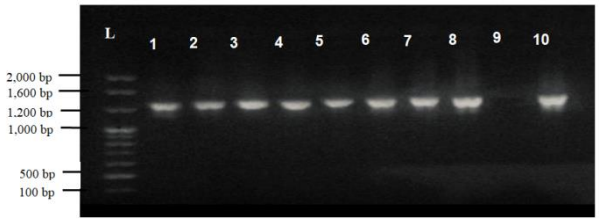

Figure: 1 Agarose gel electrophoresis (1.5%) showed a model of amplified 16S rRNA gene (1500bp). Lane L: 100 bp Marker, Lane 1-19: 16S rRNA gene bands for bacterial isolates.

Identification of bacterial species by 16 S rRNA sequencing.

All amplicons of the 16 S rRNA gene were subjected to sequence and identified ,the species were:

•Escherichia coli (n = 27 of 86 /31.3 %)
•Klebsiella pneumoniae (n = 12 / 13.9%)
•Enterobacter hormaechei (n = 8 /9.3%)
•Enterobacter cloacae (n = 5 /5.8 %)
•Shigella flexneri (n = 3 /3.4 %)
•Enterococcus faecalis (n = 3 / 3.4 %)
•Bacillus cereus (n = 3 / 3.4 %)
•Pseudomonas aeruginosa (n = 2 / 2.3 %)
•Staphylococcus gallinarum (n = 2 / 2.3 %)
•Salmonella enterica (n = 2 / 2.3 %)
•Bacillus licheniformis (n = 2 / 2.3 %)
•Proteus mirabilis (n = 2 / 2.3 %)
•Fusobacterium nucleatum, (n = 1 /1.1%)
•Enterobacter ludwigii, (n = 1 / 1.1 %)
•Staphylococcus pseudintermedius, (n = 1 / 1. 1 %)
•Lactococcus garvieae, (n = 1 / 1. 1 %)
•Escherichia fergusonii, (n = 1 / 1. 1 %)
•Citrobacter murliniae (n = 1 / 1. 1 %)
•Lactobacillus fermentum, (n = 1 / 1. 1 %)
•Providencia rettgeri, (n = 1 / 1. 1 %)
•Lactobacillus mucosae, (n = 1 / 1. 1 %)
•Lactococcus formosensis, (n = 1 / 1. 1 %)
•Bacillus paralicheniformis, (n = 1 / 1. 1 %)
•Bacillus proteolyticus, (n = 1 / 1. 1 %)
•Enterobacter mori, (n = 1 / 1. 1 %)
•Enterococcus thailandicus, (n = 1 / 1. 1 %)
•Serratia marcescens, (n = 1 / 1. 1 %)

Genetic sequencing results showed the diagnosis of 27 types of bacteria from the patients, including the Enterobacteriaceae family, where E. coli bacteria had the highest percentage among the diagnosed isolates, at 31% of the total diagnosed isolates, in addition to the presence of other intestinal types in varying proportions, the most important of which is Salmonella enterica bacteria at 2.3%. Rare genera were also diagnosed in the intestines, such as Lactococcus of two types, L. garvieae and L. fermentum at 2.2%, Escherichia fergusonii and Enterobacter mori. The anaerobic bacteria Fusobacterium nucleatum was also diagnosed at 1.1% of the total isolates, and various other pathogenic bacterial genera.

DISCUSSION

Isolation of bacteria

Most of the samples that were cultured showed good bacterial growth on all media used, and a large percentage of the isolates were pure.

16S rRNA gene amplification

The 16S rRNA gene sequence considered the 'gold standard' for bacterial identification and classification at the species level (Manjul and Shirkot, 2018) was used instead of time-consuming and potentially inaccurate biochemical tests. Molecular methods, such as 16S rRNA sequencing, offer a superior tool for bacterial classification and phylogenetic analysis.

Escherichia coli, a common bacterial species, is frequently isolated from colorectal cancer (CRC) patients. It can cause intestinal inflammation and produce toxins like colibactin, which can damage DNA and contribute to cancer development(Al-Harbi et al., 2020)..Escherichia fergusonii, while less common, is an emerging opportunistic pathogen that has been linked to various human and animal diseases, including diarrhea. Recent studies have identified its presence in individuals with esophageal, pancreatic, and colorectal cancer, suggesting a potential role in cancer development. However, the specific mechanisms by which it contributes to cancer remain unclear (Jiang et al., 2023; Zang, et al. ,2023). Klebsiella pneumoniae is a common bacterium causing infections. While not directly cancerous, it can promote tumor growth through various virulence factors, including colibactin produced by the pks cluster. This bacterium is linked to gut colonization, mucosal invasion, and serious complications like meningitis and potential tumorigenesis. It's frequently found in the stool of colorectal cancer patients. .(Chiang et al., 2021;Strakova et al., 2021). Serratia marcescens is an opportunistic bacterium

commonly found in hospital settings. It can cause gastrointestinal issues like diarrhea, highlighting its potential role in intestinal infections. (Ochieng et al., 2014). Proteus mirabilis bacteria are found in high numbers in the stool of colorectal cancer patients and inside tumor cells, suggesting a strong link between them. These bacteria may play a role in tumor cell transformation and spread. (Wachsmannova et al., 2019). Salmonella and Shigella bacteria, particularly Salmonella enterica and Shigella flexneri, are linked to colon and rectal cancer. These bacteria produce toxins like CDT, which can damage DNA and contribute to cancer development. (Cuevas-Ramos et al., 2010). Enterobacter species, including E. cloacae, E. hormaechei, E. ludwigii, and E. mori, were found in CRC patients. E. cloacae and E. hormaechei are common gastrointestinal pathogens, especially in immunocompromised individuals. E. ludwigii is an opportunistic pathogen, while E. mori is typically associated with plants but has been found in rare human infections (Hartl et al., 2019; Sánchez-Alcoholado et al., 2020). Citrobacter murliniae, a bacterium linked to various diseases and chronic irritable bowel syndrome (IBS), was found in the study. Citrobacter murliniae, a bacterium linked to various diseases and chronic irritable bowel syndrome (IBS), was found in the study. Pseudomonas aeruginosa, a common intestinal common intestinal bacillus colonizer in hospitalized CRC patients, especially those with advanced disease, was isolated from some samples. (Vuotto et al., 2013; AlRazn, and AbdulHussein, 2021). Enterococcus bacteria, especially E. faecalis, were found in CRC patients. E. faecalis is linked to CRC development through mechanisms like ROS production and DNA damage. (Yu et al., 2022; Ionescu et al., 2024). Lactobacillus bacteria, specifically L. fermentum and L. mucosae, were found in the stool samples. These bacteria are often associated with the early stages of colorectal cancer. (Kotelevets and Chastre ,2023). Lactococcus bacteria, rarely found in the intestine, was identified as two species, L. garvieae and L. fermentum were identified in CRC patient samples. This might be due to changes in the gut microbiome during tumorigenesis (Koliarakis et al., 2018). Providencia rettgeri, a bacterium associated with tumors, was found in CRC patients. It shares virulence genes with Fusobacteria and might contribute to CRC development. .(Galac and Lazzaro, 2012; Burns et al., 2015). Staphylococcus gallinarum, a pathogenic bacterium, was found in the stool samples of CRC patients (Clos-Garcia et al., 2020). Several Bacillus species, including B. cereus and B. licheniformis, were found in CRC patient stool samples. While most Bacillus species are non-pathogenic, some, like B. cereus and B. licheniformis, can cause infections. Bacillus bacteria can colonize the human gut, often due to contaminated food or hospital environments(Al-Habibi et al., 2022). Bacillus cereus is a food poisoning bacterium that can also cause hospital-acquired infections, especially in cancer patients. (Haque et al., 2021). The results of the study showed that the anaerobic bacteria Fusobacterium nucleatum was identified in samples taken from people with CRC. It is a major member of the bacteria associated with colorectal cancer, It has the ability to cause mutations in DNA through the toxin fada. but we lack a systematic and in-depth understanding of its role in the development of colorectal cancer (Ou et al., 2022).

CONCLUSIONS

The study showed the isolation and molecular diagnosis of bacteria associated with people with the disease and the role they play in causing and developing the tumor, the most important of which are Fusobacterium nucleatum, Escherichia coli, and Salmonella enterica, which contribute fundamentally to the progression of the tumor. They can also be used as a unique fingerprint in diagnosing the disease by identifying pathogenic species and variation in the microbial community inside the intestine.

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