RESEARCH ARTICLE

Escherichia coli Biomarker Types in Colorectal Cancer Patients

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ABSTRACT

Colorectal cancer is the second leading cause of cancer in the globe; increasing evidence suggests that the intestinal microbiota is a key factor associated with carcinogenesis of colorectal cancer (CRC) and colonic polyps. This study was conducted on 50 adult patients of both gender. These patients were proved colorectal cancer of different stages, and they were attending gastrointestinal center in Ramadi city, Gastroenterology and Liver Disease Center at Medical City Hospital Baghdad, during the period extended from November to February 2021. Thirty-five biopsies were taken from patients with colorectal cancer diagnosed in colonoscopy, and fifteen (15) stool specimens were taken from them. Each specimen was cultivated on blood agar, MacConkey agar, at 37°C for 24 hours. Then bacterial colonies were studied grossly, their size, color, elevation, texture, and odor; dry, stained smear was stained with Gram stain. A part of the test colony was submitted to biochemical investigation using a suitable sterile medium for each test. The bacterial diagnosis was confirmed using the VITIC system. For molecular research, *Escherichia coli* isolates were purified and maintained frozen at -20°C in a brain heart infusion broth containing 20% glycerol. Another 50 control adult, with normal colonoscopy from both genders. A stool specimen was taken from each control individually and processed as soon as possible in the same way for the patient group. Test *E. coli* isolated from both patient group and control group were tested for virulence genes different Frequency of 16S ribosomal RNA (16SrRNA) gene, cytotoxic necrotizing factor (CNF) gene, cytolethal distending toxin (CDT) gene, and polyketide synthase (PKS) genes. The Tumor Necrosis Factor (TNF) was measured in sera from patients with CRC and compared to healthy individuals. The genes implicated in carcinogenesis in *E. coli* strains, such as 16SrRNA, CNF, and CTD, were only found in test isolates of *E. coli* from patients, indicating that local *E. coli* containing such genes could become pathogens in such persons, contributing to CRC induction.

Keywords: Colorectal cancer, Cyclomodulin, *E. coli*, Tumor Necrosis factor.

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Conflict of interest: None

INTRODUCTION

Colorectal cancer is the second leading cause of cancer in the Globe. The gut microbiota appears to be a major component in the development of CRC and colonic polyps, according to growing research.[1,2] Many studies have found that CRC patients' gut microbiota balance is disrupted, as well as their fecal metabolomics.[3,4] The gut microbiota may play a role in colorectal cancer's development through a number of methods. Although *Escherichia coli* is a common bacteria, Some pathogenic strains in the human gut microbiota have gained the ability to cause chronic inflammation and/or produce toxins like cyclomodulin, which may play a role in cancer development.[5] *E. coli* strains that are pathogenic produce a variety of virulence factors, including cyclomodulins, which are cell cycle modifying toxins that lead to tumor formation. Cyclomodulins include cytotoxic necrotizing factor toxin (CNF), cycle inhibiting factor, cytolethal distending toxins (CDT), and polyketide encoded toxin (PCS), which are all generated by gram negative bacteria.[6,7] The mechanism of CRC is linked to *E. coli* B2 is a phylogenetic group. It is responsible for the production of colibactin; In eukaryotic cells, a genotoxin causes DNA damage, cell cycle arrest, mutations, and chromosomal instability.[8] Colon colonization by pathogenic *E. coli* could be another indicator of a patient's poor prognosis. This category is dominated by B2 and D2 Pylogens, particularly those that are toxigenic and possess genes coding for cyclomodulin toxins.[5,9] As a result, a microbial-based diagnostic or prognostic tool for CRC screening might be used; this study is devoted to:-

- Compare *E. coli* colonization of the colon in patients with Colorectal Cancer and normal colonoscopy individual.
- Screening for 16SrRNA genes (PCR) for colibactin (Cyclomodulin) in *E. coli* isolates from patients and control individuals.
- PCR test for CTD and CNF and PKS genes in *E. coli* isolates from patients and control individuals.

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PATIENTS AND METHODS

A total of 50 adult patients from both genders were included in this study, they were attending Oncology Center in Ramadi city, The Endoscopic division of the gastrointestinal tract at Al-Ramadi Teaching Hospital, Gastroenterology and Liver Disease Center at Medical City Hospital, Baghdad during the period extended from November to February 2021. From both genders, these patients were proved to have colorectal cancer of different stages. Inclusion criteria included patients with non-resected colorectal cancer and colon polyps. Written consent was taken from each individual included in this study. Chemotherapy, a history of previous colon resection, and antibiotic use for at least four days before to sample were all used as exclusion criteria. Thirty-five biopsy and 15 stool specimens were taken from them. Each specimen was investigated bacteriologically as so as possible, in delayed conditions, a transport medium was used. Bacteriological investigations were done at Bacteriology Lab at the Microbiology Department, College of Medicine, University of Anbar. Each specimen was cultivated on blood agar, MacConkey agar, at 37°C for 24 hours. Then bacterial colonies were studied grossly, their size, color, elevation, texture, and odor. Apart from the test colony was submitted to the biochemical investigation using a suitable sterile medium for each test. Like MRVP, urease, citrate utilization, indole, and other requested tests like metallic shine colonies on Eosin Methylene Blue to confirm *E. coli* diagnosis.[10] The bacterial diagnosis was confirmed using Vitec system.[11] *E. coli* isolates were purified and to be employed for molecular research. It was stored frozen at -20°C in brain heart infusion broth containing 20% glycerol. Three 3 mL of venous blood was taken from each patient. Aseptically, serum specimen was pooled from each blood specimen and kept frozen to be used for the serological test.

Control Group

Other adults (50) with normal colonoscopy individuals from both genders were included as control individuals; stool specimen was taken from each controlling individual. Each specimen was processed as soon as possible in the same way for the patients group. Blood specimen was taken from each individual in this group and manipulated as mentioned above in the patient's group.

Table 1: Oligonucleotide primers sequences used for PCR amplification.[12]

Primer	*Sequence (5'-3')*	*Amplicon size (bp)*
16srRNA	F: TCAAA(G,T)GAATTGACGGGGGC R: GCCCGGGAACGTATTAC	473
PKs	F: ATTCGATAGCGTCACCCAAC R: TAAGCGTCTGGAATGCAGTG	2,119
CNF	F: GGGGGAAGTACAGAAGAATTA R: TTGCCGTCCACTCTCACCAGT	1,112
CDT	F: GAAAGTAAATGGAATATAAATGTCCG R: AAATCACCAAGAATCATCCAGTTA	467

Molecular Study

Extraction and purification of genomic DNA from bacterial isolates:

The bacterial specimen was taken by loop and placed in brain heart infusion broth to do suspension and incubated at 37°C for 24 hours. then the bacterial suspension was used for DNA extraction using Promega kit. Extracted Bacterial DNA was kept frozen to be used for PCR gene study.

The PCR study was done on primers for the following *E. coli* genes mentioned in (Table 1) using PCR system and a suitable program for each gene.

Statistical Analysis

Data was analyzed using SPSS version 2.

RESULTS

E. coli was isolated from all the specimens from patients. *E. coli* showed pink-colored colonies due to lactose fermentation, and colonies showed metallic shine on Eosin Methylene Blue agar (Figures 1 and 2). Regarding the biochemical test, *E.coli* showed positive Indole, MR, and negative VP. All isolates showed positive confirmatory Vitek system results.

Detection of Virulence factors genes in *E. coli* Isolates: Virulence genes had been identified in tested *E.Coli* isolates from patients, and it was found that different Frequencies of 16SrRNA gene, CNFgene, CDT gene, and PKS genes in tested *E. coli* isolates *E. coli* showed Highest content ratio 16SrRNA

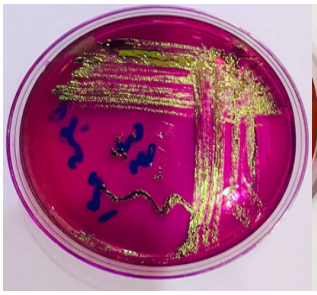

Figure 1: *E.coli* on EMB (Biopsy)

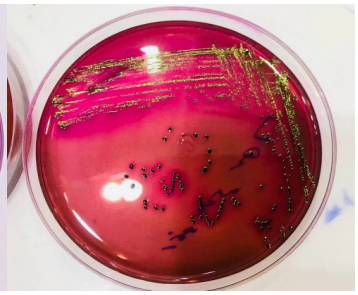

Figure 2: *E.coli* on EMB (Stool)

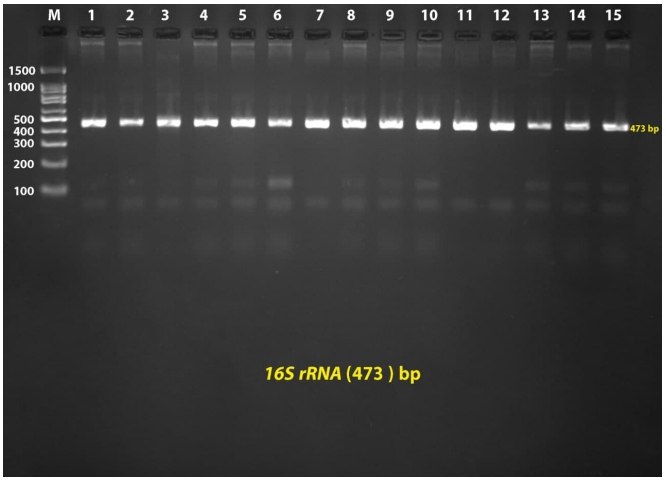

Figure 3-A: The PCR product for *E. coli* isolates from... lane (1_15), lane M ladder. The amplified *16SrRNA* gene (437 bp) electrophoresed in 2% agarose gel at 75 volt/cm for 45 min.

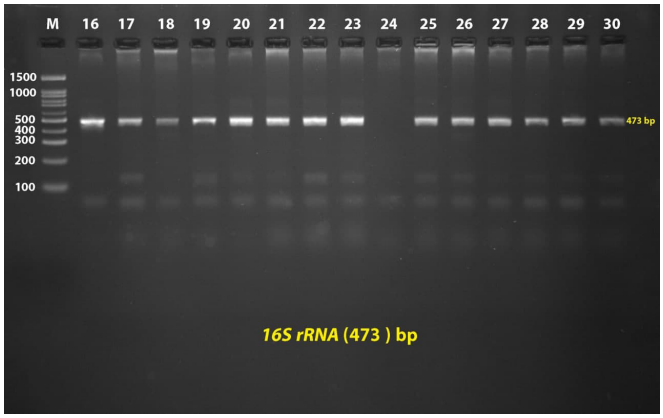

Figure 4-B: The PCR product for *E. coli* isolates from... lane (16_30), lane M ladder. The amplified *16SrRNA* gene (437 bp) electrophoresed in 2% agarose gel at 75 volt/cm for 45 minutes.

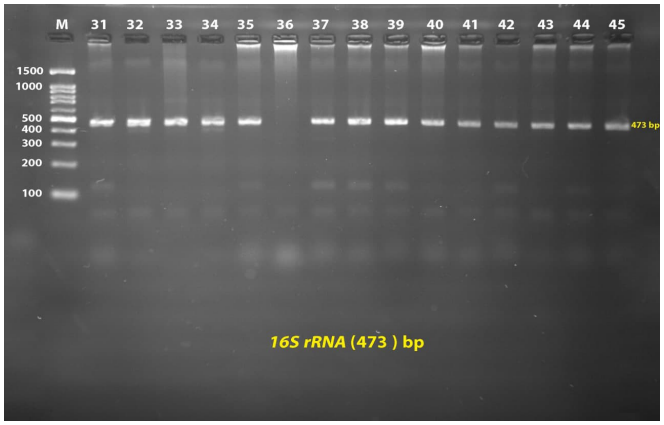

Figure 5-C: The PCR product for *E. coli* isolates from... lane (31_45), lane M ladder. The amplified *16SrRNA* gene (437 bp) electrophoresed in 2% agarose gel at 75 volt/cm for 45 minutes.

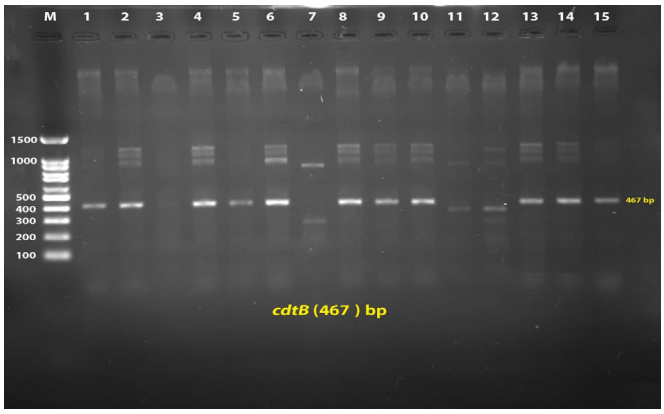

Figure 6-A: PCR product for *E. coli* isolates from lane (1_15), lane M ladder. The amplified CDT B gene (467 bp) was electrophoresed in 2% agarose gel at 75 volt/cm for 45 min.

gene (43, 80%) followed by CDT gene (35, 70%) and CNFgene (5,10%).

Control Group

E. coli was isolated from all stool specimens of the control group were, showing positive cultural, morphological, and biochemical tests mentioned for the *E. coli* isolated from

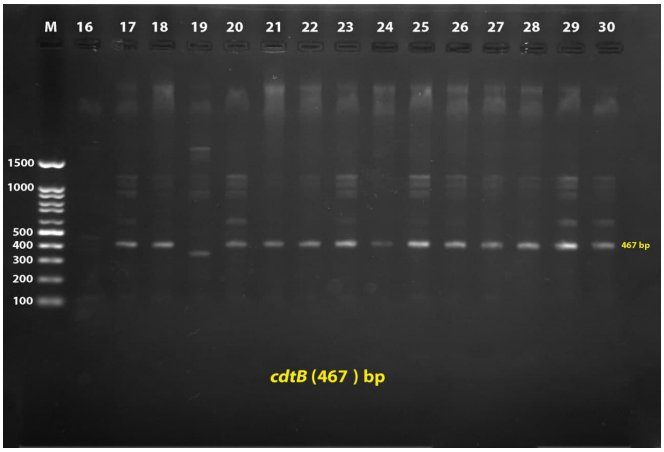

Figure 7-B: PCR product for *E. coli* isolates from, lane (17_30), lane M ladder. The amplified CDT B gene (467 bp) was electrophoresed in 2% agarose gel at 75 volt/cm for 45 min.

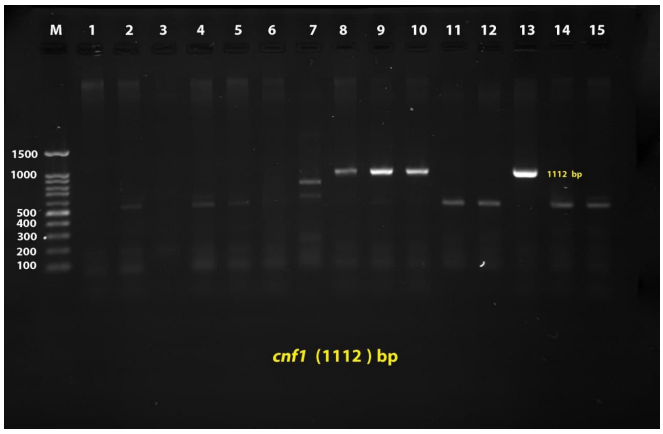

Figure 8: Lane (8_13), Lane M ladder, PCR product for *E. coli* isolates from culture. The amplified gene *cnf1* (1112 bp) was electrophoresed in a 1% agarose gel for 45 minutes at 85 volt/cm. After staining with Red Saif dye, DNA was seen by UV light.

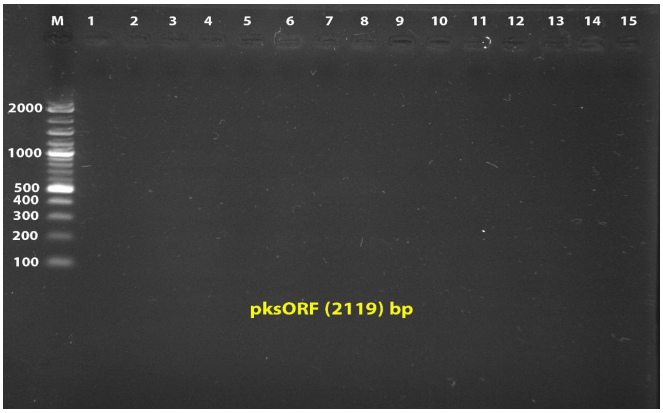

Figure 9: PCR results from patients by using agarose 2%, Red saif, (1_15), Absence of pks gene in *E. coli* isolates.

patients group specimens. But all these tested *E. coli* isolates isolated from control individuals were free of virulence genes, *16SrRNA gene, CNFgene, CDT* gene, and *PKS* genes (Figure 10).

Table 2: Frequency of 16SrRNA, CNF, CDT, and PKS genes of isolates in the samples.

	Gender								
Isolates source	*Male*	*Female*	*Urban*	*Rural*	*Total*	*PKS gene*	*CDT gene*	*CNF gene*	*16SrRNA gene*
Stool specimen	7	8	6	7	15				
Biopsy specimen	19	16	16	21	35				
Total	26	24	22	28	50	0	35	5	43
X^2					-	-	3.84	0.721	0.479
p-value					-	-	0.05 Significant p = 0.05	0.394 Non-significant p > 0.05	0.487 Non-significant p > 0.05
Risk ratio						-	*4.333*	*2.111*	*8.142*

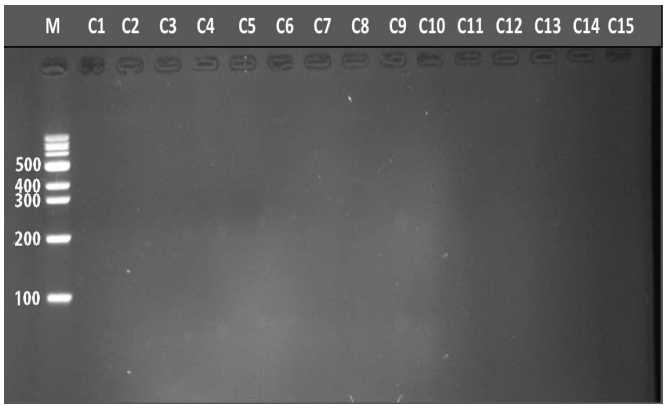

Figure 10: PCR results from control individuals by using agarose 2%, Red saif, C (1–15) control negative, bench top marker.

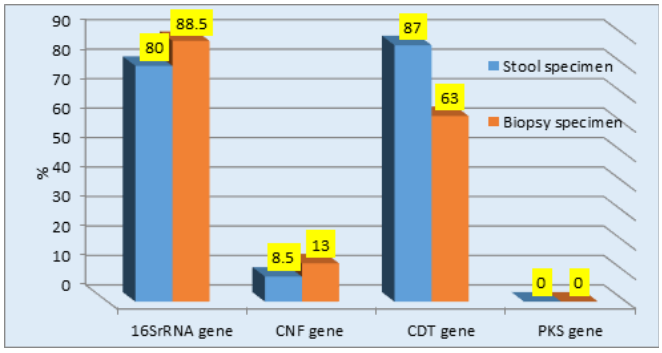

Figure 11: Graph of 16SrRNA, CNF, CDT, and PKS genes of isolates in the samples

Gene Level in Patients and Control Group

16 SrRNA genes were detected (43)، (35) for CDT، (5) for CNF of *Escherichia coli* isolates taken from patients (Table 2). Whereas all *E. coli* isolates from normal control individuals showed negative results for these genes.

TNF ELISA

The tumor necrosis factor in people with colorectal cancer was higher than in the non-affected people, where their percentage was $p < 0.05$. As shown in (Figures 12 and 13) and Table 3.

DISCUSSION

This study that was conducted showed that the highest incidence of colorectal cancer was (38%) among those belonging to the age group (55–70) years, while the disease was in other people

Table 3: ELISA TNF level in the patients and control group

Groups	*Mean*	*SD*
Control	22.44	4.27
Patients	111.28	27.95
T test value	22.23(S)	
P-value	0	

SD: Standard deviation.
S: Significant difference at $p < 0.05$

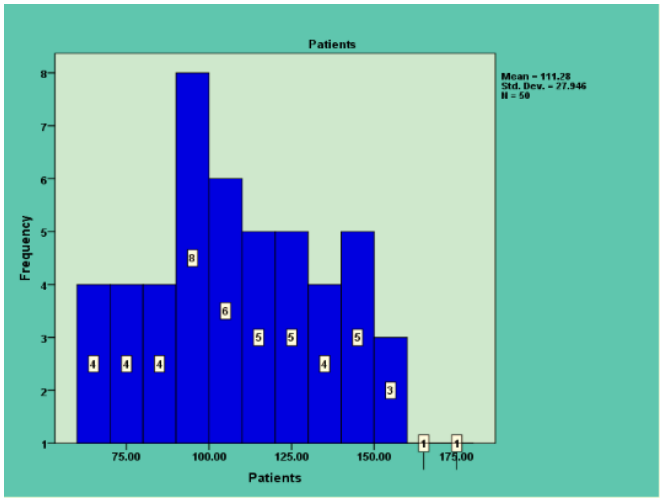

Figure 12: Graph of TNF value in the patients

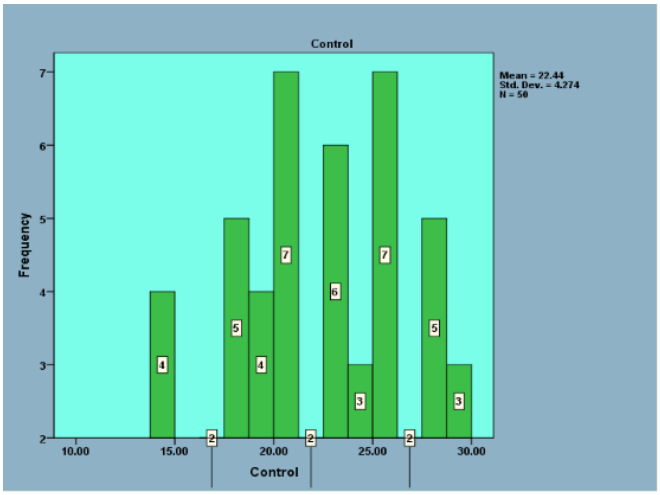

Figure 13: Graph of TNF value in the control

Table 4: Frequency of patient age of isolates in the samples

Age	*Frequency*	*Colorectal cancer*
(25 – 40)	13	
(40 – 55)	11	
(55 – 70)	19	
(70 – 85)	7	
Total	50	100%
	-	6.21
p-value	-	0.111 Non-significant p > 0.05

Table-5: Frequency of patient male and female isolates in the samples

Gender	*Frequency*	*Percent for age*
Female	24	
Male	26	
Total	50	100%
	-	0.872
p-value	-	0.241 Non-significant p < 0.05

Table 6: Frequency of patient Rural and Urban of isolates in the samples

Resident	*Frequency*	*Percent*
Rural	28	
Urban	22	
Total	50	100%
	-	2.12
p-value	-	0.0032 Significant p < 0.05

in the age group 25 to 40 years with a rate of (26%). The percentage of the age group was 40 to 55 years (11%), and the age group was 70 to 85 years (7%). As shown (Table 4). These results were non-significant X^2=6.21, p-value = 0.111, p > 0.05. This study agreed with Prashanth Rawla, and Tagore Sunkara in 2019, colorectal cancer is more common in those over the age of 50, and that the risk of having the disease increases with each decade after the age of 40. Except for those with a strong family history of the disease, these malignancies are extremely rare in people under the age of 40.[13]

There was an increase in the incidence of colorectal cancer among the young age group by 26% during this year, which could be due to the transformation of the eastern style to the western style of living, which led to an increase in the incidence of the Iraqi population.

This study showed that 26% of colorectal cancer patients are males and 24% are females, as shown in Table 5, where this result was non-significant in the incidence of colorectal cancer between women and males p < 0.05, which is consistent with a study by Alan White, Lucy Ironmonger, *et al.* in the 2018. on the incidence of colorectal cancer Colorectal cancer affects both men and women equally, but males are more prone to get it as they age due to increases in the inner walls of the colon or rectum, which are not malignant.[14] There is also a study in the United States of America in 2014 that proved the incidence of males is higher than females, as 71,830 men were diagnosed with colorectal cancer, and the percentage of females was 65,000 women.[15]

Genetic and epigenetic factors and dietary habits play a major role in the sex-specific differences in the risk of colorectal cancer. The physiological, lifestyle can also explain the observed results, and social-behavioral due to exposure to risk factors and carcinogens such as individual, history, and work in both sexes.

Our study showed that 44% of colorectal cancer patients were from urban areas and 56% from rural areas, and this result is considered significant (Table 6), where the percentage was p < 0.05. This result was agreed with Charles R. Rogers, Brenna E. Blackburn *et al*. in 2020 years.[16] There is a study conducted by G Launoy, X Le Coutour, and others in 1992, indicating that there is no significant difference between rural and urban, but the difference is the late diagnosis, especially for women in rural areas, due to lack of health. Awareness of colorectal cancer, so efforts should be made to provide medical information to women in rural areas in order to reduce delays in diagnosis and improve survival and early detection of tumors.[17] In the United States, specifically, this study by Jonathan K. *et al*. in 2014 found that colorectal cancer negatively affects rural areas and delays diagnosis in these areas.[18]

Rural patients were showing a higher rate of colorectal cancer, this was might be attributed to the exposure of rural individuals to carcinogenic materials during their life through manipulation of fertilizer, particularly nitrogen fertilizers in their agriculture and appreciation to their farms.

Consumption of green food reach with carcinogenic materials particular plants food source reach with phytoremediation plants. Lack of culture and medical indication in rural area. Social states and local happens in rural areas prevent early diagnosis of cancer females.

The PCR assay was performed to detect three genes responsible for some virulence factors, and the results showed that some isolates harbored these genes by amplifying the specific parts of these genes that can be associated with clinical diseases in the gastrointestinal tract and can cause colorectal cancer. (Cell-up toxin, cytotoxic necrosis factor, 16SrRNA Toxins, and encoded polyketide toxin) as shown in the Table 2.

This study considers that the first gene encoding cyclomodulin is CDT gene, and it was proven through the study that it is present in the *Escherichia coli* bacteria, where stool samples showed 13 positive samples to CDT, out of a total of 15 and thus considered a percentage (87%), and in biopsy samples 22 samples out of a total of 35. Its presence is 63%, where the (X^2 = 3.84), so the ratio of the total CDT gene is p value = 0.05, and thus this result is significant. Therefore, the risk ratio = 4.33, which is equal to four double the risk ratio for this gene.

This result was in agreement with a study (Vanessa Graillot, Inge Dormoy,and others in 2016) in which E.coli was examined for the bulging factor (CDT) and the virulence factors associated with this gene, where it was found that it affects the composition of human microbiota on the formation of tumors, especially in colorectal cancer. CDT displays dual DNase and phosphatase activities and induces DNA double-strand breaks, cell cycle arrest, and apoptosis in a broad range of mammalian cells. As CDT could promote malignant transformation, we investigated the cellular outcomes induced by acute and chronic exposures to *E.coli* CDT in normal human colon epithelial cells (HCECs).[19]

This result is consistent with the study (Susanna A Kurnick, Anthony J Mannion, and others in 2019) where CDT are encoded by 3 adjacent genes cdtA, cdtB, and cdtC that can be either chromosomally or plasmid-encoded. All three genes are required for the production of this heat-stable exotoxin, which bears considerable homology to DNAse I and causes DNA breaks. 13CDT have been classified into subgroups I through V21 according to variations in amino-acid sequences and genomic locations.[7]

The second gene encoding cyclomodulin is the CNF gene. This study proved that it is present in *Escherichia coli*, where it showed here stool samples (2), which equals 8.5% out of a total of 15. Biopsy samples (3) showed which equals (13%) of the total 35, i.e., its total sum for the presence of the CNF gene samples is equal to (5) out of a total of (50), i.e., the percentage of its presence (21.5%) where the (X^2=0.721), p-value = 0.394 and the risk ratio = 2.111, that is, the risk is twice. This study of this gene was identical to the study (2013), where this study confirmed that after the acquisition of virulence factors, including the protein toxin CNF1. This Rho GTPases-activating toxin induces dysfunctions in transformed epithelial cells, such as apoptosis counteraction, pro-inflammatory cytokines' release, COX2 expression, NF-kB activation and boosted cellular motility. As cancer may arise when the same regulatory pathways are affected, it is conceivable to hypothesize that CNF1-producing *E.coli* infections can contribute to cancer development.[20] In this study (2007) carried out by (Alessandro Giamboi Miraglia, Sara Travaglione, and others). It was found that the cytotoxic necrosis factor is a protein toxin produced by some pathogenic strains of *Escherichia coli* that precisely activates Rho, Rac, and Cdc42 GTPases.[21]

In our study, it showed the presence of a third gene encoding cyclomodulin present in *Escherichia coli* present in colorectal cancer patients, where the percentage positive for a gene (16SrRNA) from the total of 12 stool samples, i.e. 80% of the total (15) stool samples, and from the total biopsy samples positive for the gene (16SrRNA) is 31 positive samples, i.e. (88.5%) of the total of (35) biopsies, thus the total samples present in this gene equals (43) out of (50) stool and biopsy samples, where the ratio X^2=0.479, p-value = 0.487 and risk ratio = 8.142, That is, the risk ratio for this gene is eight double. This study was identical to the study (Julia L. Drewes, Ames R. White and others) that demonstrated the presence of this gene and its association with the gut microbiome that causes colorectal cancer.[22] There is another study for (Michael J. Cox, William O.C.M., and others) in (2013) where it proved that there is a sequence of genes, including 16SrRNA gene, responsible for the structure of the gut microbiome, and this gene is considered the first genetic sequence of colorectal cancer.[23] There is also a study to (Iradj Sobhani, Julien Tap, and others) proving that this gene consists of nine highly variable conserved regions. The 16SrRNA gene is used to identify, classify and estimate microbes within the microbial samples of the human gut.[24]

In this study, the PKS gene did not appear with a negative result in the two samples Stool and biopsy = zero, and the reason may be due to the Absence of it in this strain in the *E.coli* bacteria. This result was in contrast with (Takayuki Shimpoh, Yoshihiro Hirata, and others) (2017), where results showed that 4.3% of the group of consecutive colorectal cancer samples were (pks+E.coli) where samples were isolated from disease tissue and compared the result with healthy people.[25] There is a study by (Nicol Strakova, Kristyna Korena, and others) in (2021) that the PKS gene is found in (Klebsiella pneumoniae) bacteria, as it increases the possibility of serious complications of bacterial infection, as it was assumed that in these bacteria the gene could be a potential biomarker for the spread of bacteria. The tumor caused by this bacterium.[26]

Our study showed that the tumor necrosis factor in people with colorectal cancer was higher than in the non-affected people, where their percentage was $p < 0.05$ as shown in Table 2. This result was similar with a study conducted by (Omar A Al Obeed, Khayal A Alkhayal *et al*.) in (2014) that demonstrated that high levels can be an independent diagnostic indicator for colorectal cancer and that targeting TNF may be a predictive tool by evaluating the clinical stages of colon cancer and rectum.[27] Where the first study in the Middle East was for (Maha-Abdulla Hamadien, Zahid Khan *et al*.) in (2015), where they found that TNF contributes to inflammation and involvement in colorectal cancer, where single nucleotide polymorphism shapes (SNPs) in the TNFalpha promoter can affect colon cancer risk. and rectum by regulating TNF production.[28] There was also a study by (Sanja Kapitanović, Tamara Čačev, and others) in (2014) indicating that the role of TNF is one of the luminal-modifying genes in the development of sporadic colorectal cancer.[29] In conclusion: Genes imposed in carcinogenesis in *E. coli* strain as 16SrRNA, CNF, CTD were detected in the test isolates of *E. coli* from patients only. This give a prove that resident *E. coli* carrying such genes could be a shift into pathogens in such individuals and sharing in CRC induction in patients. The incidence of colorectal cancer according to age in young people was 26%, which is considered a high rate, unlike the basic rule of infection with age in the elderly only. The infection rate of CRC for this year was higher in males than females. Environmental factors are likely to be a causative factor for infection because there is a difference between the populations of the infected, where infections are more frequent in rural areas.

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