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Cyclomodulins, Colibactin, and Biofilm-Associated Genes in *E. coli* from Colorectal Cancer and Precancerous Lesions

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ABSTRACT

Introduction: Colorectal cancer (CRC) remains a significant global health challenge. Specific strains of *Escherichia coli* elaborating virulence factors, including cyclomodulins and colibactin, have been increasingly implicated in CRC pathogenesis. This study aimed to determine the prevalence of genes encoding these toxins, namely *cnf1*, *cdtB-I*, and *clbB*, alongside genes associated with biofilm formation, *csgA* and *flu*, in clinical *E. coli* isolates from patients diagnosed with CRC or precancerous lesions. **Methods:** A total of 44 *E. coli* isolates were obtained from colorectal tissue biopsies of patients diagnosed with CRC or precancerous polyps, and from healthy controls. PCR was employed to screen for the presence of the toxin-encoding genes *cnf1*, *cdtB-I*, and *clbB*, as well as the biofilm-associated genes *csgA* and *flu*. Biofilm formation was assessed quantitatively utilizing a standard microtiter plate assay. **Results:** The toxin-encoding genes *cnf1* and *cdtB-I* were each detected in 14 isolates (31.8%) across all study groups (CRC, polyp, and healthy controls). In contrast, the *clbB* gene was identified in 5 isolates (11.4%), exclusively within the polyp and healthy control groups. The biofilm-associated genes *csgA* and *flu* exhibited the highest prevalence, being detected in 41 (93.2%) and 22 (50.0%) isolates, respectively, across all groups. Notably, none of the tested isolates demonstrated biofilm formation capability under the experimental conditions employed. **Conclusions:** This study demonstrated the presence of the *cdtB-I* gene in *E. coli* isolates from both early-stage CRC (stages I and II), with a notably higher prevalence in stage I. Furthermore, *cdtB-I* was also detected in precancerous polyps classified as both high-grade dysplasia (HGD) and low-grade dysplasia (LGD). Intriguingly, the *clbB* gene was conspicuously absent from all CRC isolates of stages I and II. These findings suggest a potential role for *cdtB-I* in the early stages of CRC development, warranting further research to elucidate its precise impact on the progression of CRC. The presence of these virulence-associated genes, without significant differences across groups, underscores the complexity of *E. coli*'s involvement in colorectal carcinogenesis.

INTRODUCTION

Colorectal cancer (CRC) remains a significant global health burden, ranking as the third most commonly diagnosed malignancy in males and the second in females worldwide [1]. Global statistics from 2018 indicate that approximately 1.8 million new cases were diagnosed, resulting in an estimated 861,000 deaths, frequently attributed to diagnosis at advanced stages [2].

Alterations in the physiology and function of the intestinal tract are increasingly recognized as contributing factors to diseases, including CRC [3]. These conditions are often characterized by a complex interplay of structural and physiological changes, encompassing heightened visceral sensitivity, altered mucosal immunity, dysbiosis of the gut microbiota, and aberrant central nervous system processing of intestinal signals [4].

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Beyond genetic predispositions, lifestyle and environmental factors, including smoking, alcohol consumption, dietary habits, and interactions with the gut microbiota, significantly influence CRC development [5, 6]. Specifically, certain strains of intestinal *E. coli* have been increasingly implicated in oncogenesis, particularly in the context of CRC progression [7].

The gut microbiota, including commensal *E. coli*, plays a crucial role in maintaining gastrointestinal homeostasis. However, dysbiosis disrupts this delicate equilibrium, leading to a reduction in beneficial bacterial populations and a concomitant expansion of pathogenic species [8]. This microbial imbalance has been implicated in the pathogenesis of numerous diseases, underscoring the established link between microbial dysbiosis and disease pathogenesis [3].

Strains of *E. coli* associated with Crohn's disease and CRC are predominantly from the B2 phylogenetic group, which is enriched in these conditionscompared to other phylogroups [7]. These B2 strains are characterized by a higher repertoire of virulence-associated genes compared to other *E. coli* phylotypes [9]. Such strains harbor a diverse array of virulence factors capable of disrupting the intestinal microbial balance, thereby predisposing individuals to both intra- and extra-intestinal infections [10]. Among these virulence factors are toxins, including cyclomodulins (such as CDT, CNF, and Cif) and the genotoxin colibactin, which are frequently detected in isolates from CRC patients [11]. Cyclomodulins, particularly those produced by B2 strains, exert genotoxic effects on mammalian cells, contributing to DNA damage through various mechanisms [12].

Cytolethal Distending Toxin (CDT) is a secreted AB-type toxin that induces irreversible cell cycle arrest at the G2/M phase [13]. This toxin triggers DNA damage, which, if not adequately repaired, can be propagated through subsequent cell divisions, potentially contributing to oncogenesis [14].

CDT is classified into subtypes I through V, based on variations in their amino acid sequences and encoding genes [15, 16]. Each CDT variant is composed of three subunits: CdtA, CdtB, and CdtC. Among these, CdtB exhibits cytotoxic activity, functioning as a phosphodiesterase that inhibits host DNA replication. The precise roles of the CdtA and CdtC subunits are less well-defined [17].

Within the gut microbiota, certain strains of *E. coli* produce the genotoxin colibactin (Clb), a molecule implicated in inducing DNA damage, specifically double-strand breaks [18]. The *clb* genes, encoding non-ribosomal peptide synthetases responsible for colibactin biosynthesis, are clustered within a 54-kb pathogenicity island known as the *pks* island [16]. Notably, this *pks* island also confers a survival advantage by promoting bacterial persistence within the intestinal environment. The induction of these double-strand breaks by colibactin contributes to chromosomal instability, a critical step in the progression towards colorectal cancer [19]. Colonization with *pks+ E. coli* strains has been implicated in the initiation of sporadic CRC [20] and may contribute to tumor survival by inducing senescence through the secretion of growth factors [21]. Cytotoxic necrotizing factor (CNF), a 115 kDa cyclomodulin, disrupts cellular homeostasis by modulating cell cycle progression and cytoskeletal architecture through the constitutive activation of the Rho family of GTPases, leading to characteristic cellular phenotypes such as micropinocytosis, megalocytosis, and multinucleation. Encoded by the *cnf* gene, CNF is expressed in two primary isoforms: CNF1 (115 kDa) and CNF2 (110 kDa) [22]. Strains expressing CNF1 have been significantly implicated in tumorigenesis and the progression to invasive carcinoma [23], a finding corroborated by observations of elevated CNF1 expression in CRC patients [24]. These intricate virulence mechanisms underscore the complex interplay between *E. coli* and host tissues, with the potential to drive oncogenic transformations. A comprehensive understanding of these interactions necessitates a detailed mechanistic investigation into how these bacterial pathogens contribute to CRC development through their specific virulence factors.

The prevalence of genotoxin-producing *E. coli* isolates exhibits geographical variation [25]. Mounting evidence suggests a potential link between *E. coli* biofilm production and CRC initiation, compromising colonic mucosal integrity by increasing barrier permeability [26]. Building upon this, Tomkovich *et al.* (2019) further identified *E. coli* biofilm formation as a contributing factor in CRC progression [27]. In their study, biofilm formation was assessed *in vivo* by isolating biofilm-positive *E. coli* strains from healthy individuals and CRC patients and subsequently inoculating germ-free mice with these isolates. Strikingly, their findings revealed that isolates from both CRC patients and, unexpectedly, healthy individuals, induced colonic inflammation in the recipient mice [27]. Enterohemorrhagic *Escherichia coli* (EHEC) strains exhibit adherence to epithelial cells, a process mediated by a diverse array of surface appendages, including pili and fimbriae. Specifically, EHEC expresses several types of fimbriae, including curli fimbriae, F9 fimbriae, and type 1 fimbriae, as well as the surface antigen 43 (Ag43) [28]. Curli fimbriae and Ag43 both promote *E. coli* adhesion to a wide range of human cell types, thereby playing a crucial role in biofilm initiation and maturation [29, 30]. The biosynthesis of curli fimbriae is governed by two distinct operons: the *csgBA* operon, encoding the structural subunits, and the *csgDEFG* operon, encoding the CsgD transcriptional regulator and the CsgEFG export apparatus [31].

Given the capacity of *E. coli* to induce inflammation and DNA mutations in colonic epithelial cells, its influence on colorectal cancer development and-

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progression is highly probable. Consequently, this study was designed to investigate both biofilm formation and the prevalence of genes encoding key virulence factors—specifically, cytolethal distending toxin (*cdtB-I*), cytotoxic necrotizing factor (*cnf1*), colibactin (*clbB*), curli fimbriae (*csgA*), and the adhesin (*flu* or *agn43*)—in *E. coli* isolates obtained from patients with colorectal cancer, individuals with precancerous lesions, and healthy controls.

MATERIAL AND METHODS

Sample collection. After obtaining informed consent, colonic lavage fluid samples were collected from 44 participants at the Colonoscopy Center of Sayad Shirazi Hospital and the Gorgan Gastroenterology Clinic between 2021 and 2022. Concurrently, demographic data, including age, gender, history of gastrointestinal disease, and dietary habits, were also recorded.

Inclusion criteria. Participants were enrolled in the study if they presented with symptoms suggestive of CRC or had precancerous or cancerous lesions confirmed through laboratory analysis, colonoscopy, and histopathological examination, as determined by a board-certified gastroenterologist.

Exclusion criteria. Participants were excluded from the study if they had a documented history of inflammatory bowel disease (IBD), active gastrointestinal bleeding, or irritable bowel syndrome (IBS). Furthermore, individuals who had experienced an episode of acute diarrhea within the month preceding enrollment or had received antibiotic therapy within the three weeks prior to study participation were also excluded.

Bacterial culture and isolation. During colonoscopy procedures, a 2 mL aliquot of colonic lavage fluid was aspirated from each participant using a syringe attached to the endoscope, immediately subjected to flash-freezing in liquid nitrogen, and subsequently transported to the microbiology laboratory. Upon arrival, these samples were cultured on both blood agar and eosin methylene blue (EMB) agar plates and incubated at 37°C for 24 hours under aerobic conditions. Bacterial isolates were identified as *E. coli* using established biochemical assays, including triple sugar iron (TSI) agar, lysine decarboxylase, urease, and indole, methyl red, Voges-Proskauer, and citrate utilization (IMViC) tests [32]. Confirmed *E. coli* isolates were then cryopreserved at -80°C. Throughout the study, the *Escherichia coli* reference strain PTCC 1399 was included as a quality control measure.

Assessment of biofilm formation. The biofilm-forming capacity of *E. coli* isolates was assessed using the crystal violet microtiter plate assay, as previously described by Merritt *et al*. (2011) [33]. Briefly, a 30 µL aliquot of bacterial suspension was inoculated into 3 mL of sterile brain heart infusion (BHI) broth supplemented with 1% sucrose. Subsequently, 250 µL of this inoculum was transferred into each well of a sterile 96-well microtiter plate. Following incubation at 37°C for 24 h under static conditions, bacterial growth within the wells was assessed. The wells were then washed three times with phosphate-buffered saline (PBS) to remove non-adherent cells, and the remaining adherent cells were fixed with 250 µL of 96% ethanol for 20 minutes. After fixation, the ethanol was removed, and the biofilms were stained with 200 µL of 0.2% crystal violet solution for 15 minutes. Excess stain was removed by washing the wells thoroughly with deionized water, and the plates were air-dried. The biofilm-bound crystal violet was then solubilized by the addition of 200 µL of 33% glacial acetic acid to each well. Biofilm quantification was achieved by measuring the absorbance of the solubilized dye at 492 nm using a microplate reader. Each experimental condition was tested in triplicate. Biofilm production was categorized according to the following optical density (OD) thresholds: OD < 0.1, non-biofilm forming; 0.1–0.2, weak biofilm formation; 0.2–0.3, moderate biofilm formation; and > 0.3, strong biofilm formation [34]. Sterile BHI broth supplemented with 1% sucrose served as the negative control, whereas the *E. coli* reference strain PTCC 1399 was employed as the positive control for biofilm formation.

Genomic DNA extraction. Genomic DNA was extracted from bacterial isolates using the Favorgen Genomic DNA Extraction Kit (Favorgen Biotech Corp., Taiwan) according to the manufacturer's instructions and stored at -20°C for downstream analyses.

PCR amplification and detection of target genes. The presence of the *cdtB-I*, *cnf1*, *clbB*, *csgA*, and *flu* genes in the *E. coli* isolates was determined by conventional PCR. Each PCR reaction was performed in a total volume of 25 µL, containing 8.5 µL of nuclease-free water, 1 µL of each forward and reverse primer (at a final concentration of 0.4 µM each), 12.5 µL of Taq DNA Polymerase Master Mix RED (Ampliqon, Denmark), and 2 µL of template DNA. Following amplification, PCR products were separated by electrophoresis on 1.5% agarose gels containing SYBR Safe DNA Gel Stain and visualized under UV illumination.

Detailed information regarding the primer sequences and PCR cycling parameters is available in the Supplementary Material. Specifically, Table 1 and Table 2 in the Supplementary Material provide comprehensive lists of the primers used and the corresponding thermal cycling programs, respectively.

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Table 1. Oligonucleotide primer sequences used for PCR amplification

Gene	Oligonucleotide sequence (5'→3')	PCR Product Size (bp)	Ref
cnf1	F: 5′-GGG GGAAGTACAGAAGAATTA-3′	1112	[35]
	R: 5′-TTGCCGTCCACTCTCACCAGT-3′		
cdtB-I	F: 5′- CAATAGTCGCCCACAGGA-3′	414	[36]
	R: 5′-ATAATCAAGAACACCACCAC-3′		
clbB	F: 5′- GCGCATCCTCAAGAGTAAATA-3′	283	[37]
	R: 5′-GCGCTCTATGCTCATCAACC -3′		
csgA	F: 5′- ACTCTGACTTGACTATTACC -3′	200	[38]
	R: 5′- AGATGCAGTCTGGTCAAC -3′		
flu	F: 5′- GGGTAAAGCTGATAATGTCG-3′	508	[39]
	R: 5′-GTTGCTGACAGTGAGTGTGC -3′		

Table 2. Thermal cycling parameters for PCR amplification of target genes

Step	Temperature	Time	*csgA*	*flu*	*cdtB-I*	*cnf1*	*clbB*
Initial Denaturation	95°C	5 min	✓	✓	✓	✓	✓
Denaturation	95°C	30 sec	30 sec	45 sec	40 sec	45 sec	30 sec
Annealing	51.2 - 64.3°C	30 - 45 sec	51.2°C	64.3°C	54°C	53.3°C	63.3°C
Extension	72°C	30 sec	30 sec	45 sec	40 sec	45 sec	30 sec
Final Extension	72°C	5 min	✓	✓	✓	✓	✓
Cycles			35	35	35	35	35

Statistical analysis. Statistical analyses were performed using SPSS software (version 24.0; IBM Corp., Armonk, NY, USA). Categorical data were analyzed using the chi-square (χ^2) test. Statistical significance was defined as a *P*-value of less than 0.05.

Ethical considerations. This study received ethical approval from the Ethics Committee of Golestan University of Medical Sciences (approval code: IR.GOUMS.REC.1400.122). All participants provided written informed consent prior to their inclusion in the study.

RESULTS

Study population. A total of 44 *E. coli* isolates were recovered from the colonic secretions of 44 participants, comprising 24 healthy individuals, 6 patients diagnosed with colorectal cancer (CRC), and 14 individuals with colorectal polyps. The study cohort consisted of 25 females (56.8%) and 19 males (43.2%), with participant ages ranging from 18.2 to 79.5 years. Among the CRC patients, 4 (9.1%) were classified as stage I and 2 (4.5%) as stage II according to the American Joint Committee on Cancer (AJCC) staging system [35]. Within the group of individuals with polyps, 10 (22.7%) presented with low-grade dysplasia (LGD) and 4 (9.1%) with high-grade dysplasia (HGD) (refer to Table 3).

Prevalence of virulence-associated genes in *E. coli* isolates. Analysis of the 44 *Escherichia coli* (*E. coli*) isolates from patients with colorectal cancer (CRC), individuals with polyps, and healthy controls revealed the following overall prevalence of virulence-associated genes: *cnf1*, 14 (31.8%); *clbB*, 5 (11.4%); *cdtB-I*, 14 (31.8%); *csgA*, 41 (93.2%); and *flu*, 22 (50.0%) (refer to Table 4). When considering the CRC group in isolation, the prevalence of these genes was: *cnf1*, 1 (16.6%); *clbB*, 0 (0.0%); *cdtB-I*, 4 (66.6%); *csgA*, 6 (100.0%); and *flu*, 4 (66.6%).

The *csgA* and *flu* genes were the most frequently detected among the *E. coli* isolates obtained from the colonic secretions of the study participants, while the *clbB* gene exhibited the lowest prevalence (refer to Table 4 and Figure 1).

Statistical analysis employing chi-square (χ^2) tests indicated no statistically significant association between participant gender and the presence of the *cdtB-I*, *cnf1*, *clbB*, *csgA*, or *flu* genes within the study cohort ($P > 0.05$; see Table 5). While the observed prevalence of these genes was numerically higher in female participants, this difference did not reach statistical significance, a finding potentially influenced by the slightly larger proportion of females in the study population (25 females vs. 19 males).

Statistical analyses revealed no significant correlations between the presence of the *cdtB-I*, *cnf1*, *clbB*, *csgA*, and *flu* genes in the *E. coli* isolates derived from intestinal secretions and the participants' demographic characteristics, including sex, age, family history of cancer or intestinal diseases, and dietary habits (dairy consumption, fast food consumption, vegetable consumption) ($P > 0.05$; Table 6). Moreover, the prevalence of these genes did not differ significantly among healthy individuals and those diagnosed with CRC or polyps ($P > 0.05$).

Assessment of biofilm formation capacity. None of the *E. coli* isolates, regardless of their origin from healthy controls, individuals with polyps, or patients with CRC, demonstrated biofilm formation *in vitro* when assessed using the microtiter plate assay.

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Table 3. Demographic and clinical characteristics of study participants

Category	Number of subjects	Details	Count (%)
Total participants	44		
Clinical group			
Healthy controls	24		54.5%
CRC patients	6		13.6%
Stage of CRC		Stage I	4 (9.1%)
		Stage II	2 (4.5%)
Intestinal polyp patients	14		31.8%
Type of polyp		LGD	10 (22.7%)
		HGD	4 (9.1%)
Sex		Female	25 (56.8%)
		Male	19 (43.2%)
Age		Range (Years)	18.2 – 79.5
History of specific intestinal disease		Yes	16 (36.4%)
		No	28 (63.6%)
History of cancer		Yes	6 (13.6%)
		No	38 (86.4%)
Family history of CRC		Yes	9 (20.5%)
		No	35 (79.5%)
Anemia		Yes	12 (27.3%)
		No	32 (72.7%)
Diarrhea		Yes	6 (13.6%)
		No	38 (86.4%)
Presence of blood in stool		Yes	11 (25.0%)
		No	33 (75.0%)
Constipation		Yes	15 (34.1%)
		No	29 (65.9%)
Unexplained weight loss		Yes	8 (18.2%)
		No	36 (81.8%)

Abbreviations: LGD: Low-grade dysplasia stage; HGD: High-grade dysplasia stage

Table 4. Prevalence of target genes in *E. coli* isolates stratified by participant group

Gene	Total positive (%)	Healthy controls (n=24)	Polyp patients (n=14)	CRC patients (n=6)
cnf1	14 (31.8%)	9 (37.5%)	4 (28.6%)	1 (16.7%)
clbB	5 (11.4%)	3 (12.5%)	2 (14.3%)	0 (0.0%)
cdtB-I	14 (31.8%)	8 (33.3%)	2 (14.3%)	4 (66.7%)
csgA	41 (93.2%)	21 (87.5%)	14 (100.0%)	6 (100.0%)
flu	22 (50.0%)	8 (33.3%)	10 (71.4%)	4 (66.7%)

Note: Percentages for healthy controls, polyp patients, and CRC patients represent the proportion of isolates positive for each gene within their respective groups.

Table 5. Distribution of gene positivity stratified by participant gender

Gene	Female (n=25, 56.8%)	Male (n=19, 43.2%)	*P*-value
cnf1	10 (40.0%)	4 (21.1%)	>0.05
clbB	3 (12.0%)	2 (10.5%)	>0.05
cdtB-I	9 (36.0%)	5 (26.3%)	>0.05
csgA	22 (88.0%)	19 (100.0%)	>0.05
flu	13 (52.0%)	9 (47.4%)	>0.05

Note: *P*-values were derived from chi-square (χ^2) tests. No statistically significant differences in gene frequency were observed between genders ($P > 0.05$).

Table 6. Association between symptom history and gene positivity in study participants

Symptom history	Gene positivity	*cnf1*	*clbB*	*cdtB-I*	*csgA*	*flu*	*P*-value
History of specific intestinal disease	Yes	5 (35.7%)	3 (60.0%)	7 (50.0%)	5 (35.7%)	7 (31.8%)	>0.05
History of cancer	Yes	3 (21.4%)	2 (40.0%)	1 (7.1%)	3 (21.4%)	3 (13.6%)	>0.05
Family history of colon cancer	Yes	4 (28.6%)	1 (20.0%)	0	4 (28.6%)	3 (13.6%)	>0.05
Anemia	Yes	4 (28.6%)	4 (80.0%)	6 (42.9%)	3 (21.4%)	7 (31.8%)	>0.05
Diarrhea	Yes	2 (14.3%)	2 (40.0%)	1 (7.1%)	2 (14.3%)	3 (13.6%)	>0.05
Presence of blood in stool	Yes	2 (14.3%)	1 (20.0%)	3 (21.4%)	4 (28.6%)	7 (31.8%)	>0.05
Constipation	Yes	3 (21.4%)	3 (60.0%)	5 (35.7%)	2 (14.3%)	11 (50.0%)	>0.05
Unexplained weight loss	Yes	2 (14.3%)	3 (60.0%)	6 (42.9%)	2 (14.3%)	5 (22.7%)	>0.05

Note: *P*-values were derived from chi-square (χ^2) tests. No statistically significant correlations were observed between the presence of the indicated genes and the reported symptom histories ($P > 0.05$).

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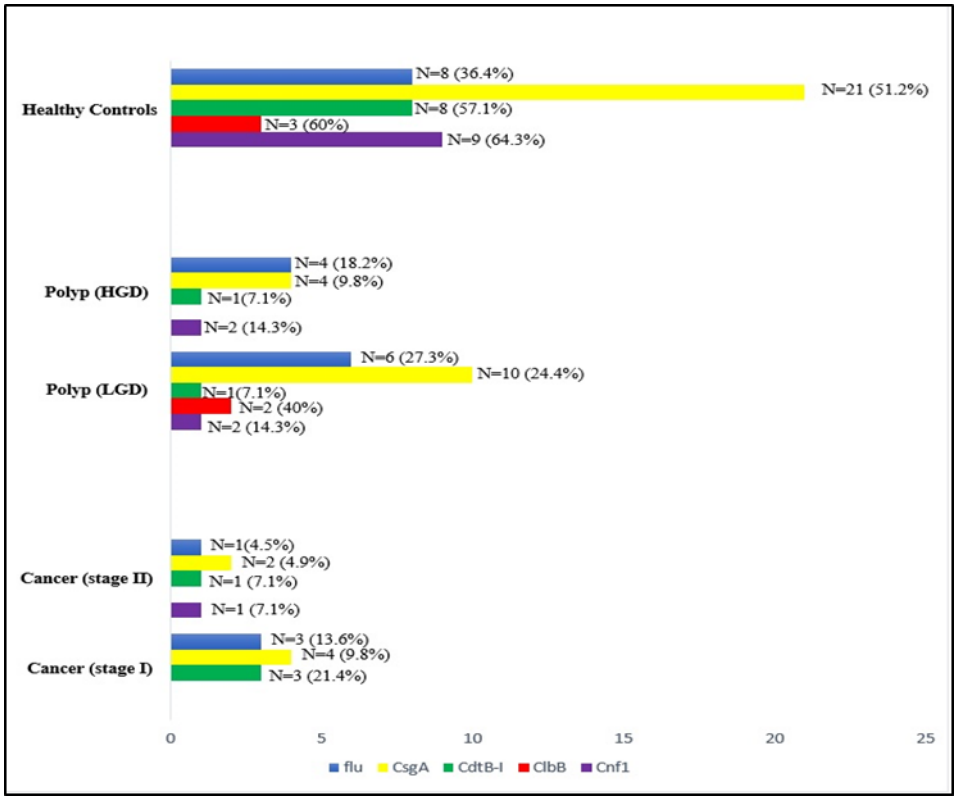

Fig. 1. The bar graph illustrates the prevalence of the *cnf1*, *clbB*, *cdtB-I*, *csgA*, and *flu* genes in *E. coli* isolates obtained from healthy controls, individuals with colon polyps (further categorized as LGD or HGD), and patients with CRC (classified as stage I or II).

DISCUSSION

The incidence of CRC has increased globally in recent decades, with a disproportionate number of cases reported in low- and middle-income countries, particularly within Asia [10]. While the etiology of CRC is multifactorial, accumulating evidence implicates specific strains of *E. coli* as potential contributors to its development [37]. Chronic intestinal inflammation can disrupt the delicate balance of the gut microbiota, leading to gastrointestinal dysbiosis and potentially fostering the expansion of certain *E. coli* strains. These pathobionts possess the capacity to induce oncogenic transformation in intestinal epithelial cells [38]. Notably, strains belonging to the B2 phylogroup frequently harbor virulence genes associated with intestinal infections and CRC [9]. These pathogenic *E. coli* strains elaborate a diverse array of virulence factors, including cyclomodulins such as cytolethal distending toxin (CDT) and cell division inhibitor factor (CdiF) (formerly known as cycle inhibiting factor), as well as the genotoxin colibactin [12]. Furthermore, a subset of these strains exhibits the capacity for biofilm formation [29]. Mucosa-associated *E. coli* strains, particularly those expressing cyclomodulins, demonstrate enhanced colonization of the gut mucosa in patients with CRC compared to individuals with healthy mucosa [39]. A growing body of epidemiological evidence suggests an association between CNF1 and IBD as well as CRC [40]. Mechanistically, CNF1 disrupts cell cycle progression by inhibiting the CDK1-cyclin B1 complex, resulting in cell cycle arrest at the G2/M transition [41]. Similarly, CDT induces cell cycle arrest and promotes genomic instability by triggering DNA double-strand breaks, ultimately leading to apoptosis [42]. The DNA damage inflicted by CDT-producing bacteria on host cells is hypothesized to contribute to oncogenesis [14]. Beyond its role in promoting colonization of host tissues, CDT can also subvert host immune responses, thereby potentially exacerbating the severity of infection [43]. Analogously, certain *E. coli* strains synthesize colibactin, a genotoxic hybrid molecule composed of polyketide and peptide moieties. Colibactin induces DNA damage through alkylation and the formation of double-strand breaks, preferentially targeting genomic loci frequently mutated in CRC [44].

In the present study, we investigated the prevalence of genes encoding *cnf1* and *cdtB-I*, the *clbB*, *csgA* and *flu*, comparing their distribution among patients with colorectal cancer, individuals with precancerous colorectal polyps, and healthy controls. Our analysis focused on comparing the distribution of these genes among patients with colorectal cancer, individuals with precancerous colorectal polyps, and a cohort of healthy controls. From the 44 colonic lavage fluid samples analyzed, *E. coli* was successfully isolated from all samples. Specifically, 20 of these samples originated from individuals with gastrointestinal conditions, comprising 14 (31.8% of the total cohort) with intestinal polyps and 6 (13.6% of the total cohort) with CRC. Martin *et al.* (2004)

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identified invasive *E. coli* in 57% of 21 CRC biopsy samples [45], while Buc *et al.* (2013) detected *E. coli* in 38 colon cancer samples [39]. More recently, Nori *et al.* (2021) reported that 23% of samples from 50 CRC biopsies contained enteropathogenic *E. coli*, predominantly belonging to the B2 phylogroup [46], and Hassan *et al.* (2023) found *E. coli* in 32% of 51 colon cancer samples [47]. Our results are consistent with these previous investigations, as we successfully isolated *E. coli* from every tissue sample of cancer and polyps examined in this study.

These studies, including the present investigation, collectively highlight the increased prevalence of *E. coli* in CRC samples. A potential mechanism underlying this elevated presence in patients with colon cancer or conditions such as polyps or IBD may involve alterations in mucosal receptor expression, which could facilitate enhanced bacterial colonization of the intestinal lining [48].

In the present study, analysis of 44 *Escherichia coli* (*E. coli*) isolates derived from human colonic secretions revealed the *cnf1* gene in 31.8% (14 isolates), with a distribution of 16.7% in CRC patients, 28.6% in individuals with intestinal polyps, and 37.5% in healthy controls. Similarly, the *cdtB-I* gene was detected in 31.8% of the isolates, distributed as 66.7% in CRC patients, 14.3% in polyp patients, and 33.3% in healthy controls. Comparative analyses with previous research reveal varying frequencies of these genes. Buc *et al.* (2013) reported a *cnf1* prevalence of 39.5% in 38 CRC samples [39], while Toth *et al.* (2003) detected *cdtB-I* in only one out of 112 intestinal *E. coli* isolates [49]. Dubois *et al.* (2010) found *cdtB* in 4% and *cnf1* in 37% of 197 isolates, predominantly from the B2 phylogroup [15], and Moreno *et al.* (2014) identified *cnf1* in just one of 41 isolates, with no *cdt* genes detected [50].

In the present cohort, the *cnf1* gene was detected in a single *E. coli* isolate from a patient with stage II colorectal cancer (CRC), which also carried the *cdtB-I* gene. This finding contrasts with the study by Nouri *et al.* (2021), who reported a *cnf1* prevalence of 16.7% (4 out of 24 isolates) in their CRC cohort, and observed an absence of the *cdtB-I* gene [46]. In our investigation, the *cdtB-I* gene was identified in six isolates: four derived from CRC patients (66.7% of the CRC isolates) and two from patients with polyps (14.3% of the polyp isolates). Among the CRC isolates harboring these genes, three were from patients with stage I disease, and one was from a patient with stage II disease; this latter isolate was the only one to carry both the *cnf1* and *cdtB-I* genes.

In this study, the *cdtB-I* gene was detected in two isolates originating from polyp samples: one from a patient with HGD and one from a patient with LGD. The relatively low prevalence of both *cdtB-I* and *cnf1* observed in our cohort is consistent with the findings of previous studies discussed earlier in this manuscript**.**

Prior investigations have often lacked detailed information regarding the specific stages of CRC in their study populations and have not extensively explored the correlation between the prevalence of the *cnf1* and *cdtB* genes and patient demographics. Despite the documented presence of *E. coli* harboring the *cnf1* gene in CRC patients, the precise mechanisms by which the CNF1 toxin influences disease progression remain largely unexplored, as highlighted in the existing literature [51].

Iyadorai *et al.* (2020) reported the presence of *pks+ E. coli* in 8 out of 48 CRC samples (16.7%) [52]. Similarly, Kosari *et al.* (2020) identified the *clbB* gene in 23.3% of 13 CRC isolates [53]. Gomez-Moreno *et al.* (2014) observed a 20% prevalence of *pks+* strains among 41 *E. coli* isolates [50], while Suresh *et al.* (2018) detected *pks+* genes in 7.6% of 462 enteropathogenic *E. coli* isolates [54]. In contrast, Shimpoh *et al.* (2017) reported a higher prevalence of *pks+ E. coli*, finding it in 43% of CRC samples and 51% of intestinal adenoma samples, based on an analysis of 72 isolates [55]. More recently, Hassan *et al.* (2023) noted an 80% frequency of *pks+ E. coli* in 10 CRC isolates [47], and Miyasaka *et al.* (2024) found *pks+ E. coli* in 65.6% of 413 CRC tumor tissue samples, predominantly in stage 0-I [56]. He *et al.* (2024) documented the presence of *pks+ E. coli* in 46% of 50 CRC samples, with 18% of these being at stage I [57].

In the present study, the *clbB* gene, indicative of colibactin production, was detected in 11.4% of the total *E. coli* isolates analyzed. Notably, none of the six *E. coli* isolates derived from CRC samples harbored this gene. Among the isolates from polyp samples, only two out of fourteen (14.3%) were positive for *clbB*, representing 40.0% of all *clbB*-positive isolates. The prevalence of *clbB* was comparatively lower in the remaining samples. While numerous studies have investigated the association between the intestinal microbiota and CRC progression [58], the precise contribution of *pks+ E. coli* to tumor growth remains an area requiring further investigation.

Our analysis revealed no statistically significant correlations between the prevalence of the *cnf1*, *cdtB-I*, and *clbB* genes and demographic factors such as age, sex, family history of cancer, history of intestinal diseases, or dietary habits across all participant groups (healthy controls, cancer patients, and polyp patients). Furthermore, the presence of these genes did not differ significantly between patients with colorectal lesions and healthy controls. *E. coli* possesses the capacity for biofilm formation, a process often mediated by genes such as *csgA* and *flu*, which encode for curli fimbriae and antigen 43, respectively-both critical components for biofilm development. In the present cohort, the *csgA* gene was highly prevalent, detected in 93.2% of the 44 *E. coli* isolates analyzed, while the *flu* gene was present in 50% of these isolates. Remarkably, all 20 *E. coli* isolates derived from CRC and polyp samples harbored the *csgA*

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gene, with 14 of these isolates also carrying the *flu* gene. Wang *et al.* (2016) reported that all 22 human *E. coli* isolates carried the *csgA* gene, with 21 of these isolates also possessing the *flu* gene; notably, these isolates exhibited moderate to strong biofilm formation capabilities [59]. Similarly, Taghadosi *et al.* (2017) identified the *flu* gene (encoding antigen 43 [Ag43]) in five human *E. coli* isolates, all of which formed strong biofilms [60]. Biscola *et al.* (2011) found that among 51 *E. coli* isolates, 65% harbored the curli gene (*csgA*) and 47% carried the *flu* gene, contributing to biofilm formation in 17 of these isolates [61]. In contrast to these findings, the present study observed that despite the high prevalence of the *csgA* and *flu* genes in *E. coli* isolates from CRC and polyp samples, as well as in the healthy control group, none of these isolates formed detectable biofilms under the tested *in vitro* conditions. This discrepancy underscores the intricate nature of biofilm formation, a multifactorial process influenced by a myriad of environmental cues and governed by complex regulatory signaling pathways [62]. In this study, we employed the crystal violet microtiter plate assay to assess biofilm formation in the isolated *E. coli* strains. However, it is important to acknowledge that this technique possesses inherent limitations that may have contributed to the lack of detectable biofilm formation. Specifically, the washing steps inherent in the microtiter plate assay can potentially dislodge weakly adhered biofilms, leading to an underestimation of biofilm-forming capacity [63]. Furthermore, the sensitivity of the microtiter plate assay is generally higher at high cell densities, potentially making it less suitable for investigating the early stages of biofilm development [63]. Microtiter plate assays, and similar *in vitro* biofilm systems, are widely utilized for studying biofilm formation; however, these methods often fall short of fully replicating the complex and dynamic *in vivo* environment [64]. The capacity of *E. coli* to form biofilms under static *in vitro* conditions can vary considerably among different strains and is significantly influenced by the specific cultivation environment [34]. This inherent discrepancy between *in vitro* phenotypic assays and *in vivo* biofilm dynamics may offer a plausible explanation for the absence of observed biofilm formation in the *E. coli* isolates examined in this study [34]. Research suggests that Ag43 plays a role in the initial stages of biofilm formation; however, its presence does not appear to significantly impact the overall biomass of the mature biofilm [65]. Furthermore, while the expression of curli fimbriae varies among different *E. coli* strains, it is not universally required for biofilm formation [66]. The intricate process of biofilm development in *E. coli* is governed by a complex interplay of numerous genes and is susceptible to modulation by a diverse array of environmental factors [67]. The study of biofilms is further complicated by the inherent limitations of current *in vitro* models and the methodologies employed to elucidate the role of specific genes in biofilm formation [68]. Reflecting the intricate and multifactorial nature of biofilm formation, our analysis revealed no significant correlations between participant demographics and the presence of the genes *csgA* and *flu*.

In conclusion, CRC poses a significant and multifactorial health challenge. Growing evidence implicates the gut microbiome, including specific bacteria such as *E. coli*, in CRC pathogenesis. This study investigated the prevalence and potential co-occurrence of cyclomodulin, colibactin, and biofilm-formation genes in *E. coli* isolates derived from intestinal secretions of individuals diagnosed with CRC, precancerous polyps, and healthy controls. Crucially, our findings did not demonstrate any significant associations between the presence of these bacterial virulence factors and participant demographics or disease status. Furthermore, the prevalence of the investigated genes did not differ significantly between samples obtained from healthy individuals and those from patients with colorectal neoplasia. Deciphering the precise contribution of toxigenic *E. coli* strains to CRC development presents a considerable challenge, underscoring the need for well-powered, prospective cohort studies. Our study was constrained by limitations inherent in patient recruitment, particularly in enrolling individuals across the spectrum of disease stages, and in achieving an optimal sample size. Therefore, future research endeavors should prioritize the recruitment of a larger and more representative participant cohort to more definitively elucidate the complex interplay between *E. coli* and CRC pathogenesis.

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Ailar Jamalli conceptualized the study and secured funding. Data curation and methodology were performed by Taghi Amiriani and Behnoush Khasheii. Formal analysis and software utilization were conducted by Mousa Ghelichi-Ghojogh and Behnoush Khasheii. Investigation and resource acquisition were undertaken by Behnoush Khasheii. Project administration was managed by Ailar Jamalli, and overall supervision was provided by both Ailar Jamalli and Samin Zamanii, with Samin Zamanii contributed to supervision. Validation was carried out by Ailar Jamalli and Samin Zamanii. Visualization was performed by Ailar Jamalli. Behnoush Khasheii drafted the original manuscript, while review and editing were contributed by both Behnoush Khasheii and Ailar Jamalli.

CONFLICTS OF INTEREST

The authors declare that there are no conflicts of interests associated with this manuscript.

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