In the prolonged human battle against colorectal cancer, could genotoxic *Escherichia coli* be in the front line of the war?

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Objective
To find an association between colorectal cancer (CRC) and genotoxic *Escherichia coli* isolated from the human microbiota.
Patients and methods
A total of 150 patients (65 males and 85 females) were recruited from the surgical endoscopy unit in the Medical Research Institute Hospital, Alexandria, Egypt. They were divided into two groups: group I included 100 neoplastic patients (benign and malignant), and group II included 50 nonneoplastic patients. After DNA extraction, real-time PCR was performed to detect the presence of *pks island* in *E. coli* strains.
Result
The number of males (57.4%) among patients with malignant neoplastic illness was higher than the number of females (42.6%). Overall, no statistical difference was observed between the studied groups regarding sex (P=0.059). Malignant neoplastic diseases were more common in patients above 45 years. Old age in malignant patients showed a high statistical significance compared with nonmalignant patients (P≤0.001). The *pks+E. coli* was detected in colorectal biopsies using the *clbB* gene as a surrogate marker of the whole *pks* island. The current study demonstrated that the prevalence of *pks*-positive *E. coli* was significantly higher in patients with malignant neoplastic disease than benign neoplastic and nonneoplastic ones (P=0.002).
Conclusion
pks+E. coli may act as a tumor promoter for CRC and could be used as a predictive marker for CRC development. In addition, the *pks+E. coli* molecular identification may be of a good value in decreasing missed lesions during conventional colonoscopy.

Keywords:
colorectal cancer, genotoxic *E*scherichia *coli*; microbiota

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Introduction

Most colorectal cancers (CRC) are sporadic, and numerous studies have suggested that alteration in the gut microbiota has been linked to CRC development [1]. CRC is the third most common cause of cancer death [2]. In addition to known risk factors such as high-fat diets and alcohol consumption, microbiota and their products could participate in initiation and progression of sporadic colon cancer by driving a variety of mechanisms, including the induction of proinflammatory and procarcinogen pathways in epithelial cells, and the production of genotoxins and reactive oxygen species. In CRC, various bacteria have been associated with carcinogenesis, including *Streptococcus bovis*, *Enterococcus spp.*, *Helicobacter pylori*, *Enterotoxigenic Bacteroides fragilis*, and toxigenic *Escherichia coli* [3].

Genotoxic intestinal *E. coli* strains producing colibactin are proposed to play a role in colon cancer development [4]. These toxigenic *E. coli* strains are able to induce a specific cytopathic effect known as megalocytosis when in contact with mammalian cells, and this effect is dissimilar to other known toxins produced by *E. coli* such as cytolethal distending toxins and cytotoxic necrotizing factor [5].

Previous studies have demonstrated that the genes involved in cytopathic effect are found in a specific genomic island. This island, termed the polyketide synthase (Pks) island, is responsible for the expression of peptide-polyketide hybrid genotoxic-cyclomodulin referred to as colibactin [5].

Colibactin is a natural and genotoxic chemical compound that is synthetized by polyketide synthases. It induces DNA double-strand breaking, DNA cross-links, chromosome aberrations, and cell cycle arrest in the G2/M phase [4]. Interestingly, pks-harboring *E. coli* strains have been isolated from intestinal microbiota as commensal bacteria and in infectious diseases such as septicemia, newborn meningitis, and urinary tract infections. In addition, colibactin-producing *E. coli* stains are over-represented in CRC, and they increase the number of tumors in various CRC mouse models. This finding of a direct induction of DNA cross-links by a bacterium should facilitate delineating the role of *E. coli* in colon cancer and engineering new anticancer agents [6,7].

The purpose of this study was to determine the association between the CRC and the pks-positive *E. coli* stains, which is responsible for the production of the colibactin toxin. The prevalence of this *E. coli* strain will be investigated in both neoplastic patients and nonneoplastic patients.

Patients and methods

Patients who had undergone colonoscopy at the Endoscopy Unit in the Medical Research Institute Hospital, Alexandria University, Egypt, in the period from October 2020 until December 2021 were enrolled in the study. Medical Research Ethical Committee approval was obtained, and a written consent was taken from all participants.

During colonoscopy, two biopsy samples were taken from each person. One was sent to the microbiology laboratory in the Medical Research Institute and the other was sent to the pathology laboratory. Based on the pathology report, 100 neoplastic patients (benign and malignant) and 50 nonneoplastic individuals (inflammatory and noninflammatory) were included as the patients of the present study. No remarkable findings were found in the noninflammatory study group.

Tissue sampling and processing

Fresh biopsy samples from patients who had undergone colonoscopy were collected in a 2-ml sterile Eppendorf filled with 200 µl of phosphate buffer saline and were kept directly in the fridge at 2–8°C.

Overall, 25 mg of the stored tissue biopsy for each study participant was transferred to another 2-ml sterile Eppendorf tube containing 80 µl phosphate buffer saline and then stored at −20°C.

DNA extraction from colonic biopsy samples

The total bacterial DNA was extracted using QIAamp DNA Mini Kit Qiagen (Düsseldorf, Germany). The QIAamp DNA purification procedure comprises 4 steps and is carried out using QIAamp Mini spin columns.

Molecular analysis

Syber Green Real-Time PCR was used to detect different genes in DNA of the extracted samples. To assess total bacterial DNA, primers were used to amplify 16S rRNA genes in biopsy samples.

clbB gene was amplified as a marker for the presence of the *pks* island. To use *clbB* as a surrogate marker for the whole *pks* island, *clbA* and *clbQ* genes were examined as supplemental *pks* island markers, which are located very near to the 5′ and 3′ terminals of the *pks* island, respectively.

Primer sequences used in PCR were as follows:

16S rRNA gene forward primer (5′-GGTGAATACGTTCCCGG-3′) and reverse primer (5′-TACGGCTACCTTGTTACGACTT-3′) [8].

clbB gene: forward primer (5′-GCGCATCCTCA AGAGTAAATA-3′), and reverse primer (5′-GCGC TCTATGCTCATCAACC-3′) [9].

clbQ gene forward primer (5′-GCACGATCGGAC AGGTTAAT-3′), and reverse primer (5′-TAG TCTCGGAGGGATCATGG-3′) [10].

clbA gene forward primer (5′-AAGCCGTATCCT GCTCAAAA-3′) and reverse primer (5′-GCTTC TTTGAGCGTCCACAT-3′) [10].

PCR reaction mixture and thermal profile

The final volume of the PCR reactions was 20 µl, which consisted of 0.6 µl (10 pmol) of each primer solution (forward and reverse), 10 µl of the MAXIMA SYBR Green Master, 6 µl of extracted DNA, and 2.8 µl of distilled water were added to bring the reaction to the final volume.

AmpliTaq activation time was 10 min at 95°C, followed by 45 cycles of PCR amplification, including denaturation for 15 s at 95°C, annealing at 55°C for 30 s and extension at 72°C for 30 s. A melting curve analysis was done to determine the purity and specificity of the amplification product. The melting curve analysis profile was 95°C for 1 min, then 55°C for 30 s, and finally, 95°C for 30 s.

Results

Pathological diagnosis and demographic data

Depending on the pathological diagnosis, patients were divided into two groups: group I included 100 neoplastic patients, and group II included 50 nonneoplastic patients. The neoplastic group was subdivided into 46% benign and 54% malignant patients. The nonneoplastic group was subdivided into 40% noninflammatory control group, 40% inflammatory non-Inflammatory Bowel Disease (IBD) patients, and 20% patients with IBD. Concerning sex, no statistically significant difference was observed among the study groups (P=0.059). Regarding patients' age, which ranged from 18 to 98 years old, there was a higher incidence of malignant neoplastic diseases in patients above 45 years old. The age of malignant patients was significantly higher than nonmalignant patients (P<0.001).

Results Keywords of molecular analysis

16S rRNA was amplified in all participants. Then, *pks* island was identified by amplifying *clbB*, *clbQ*, and *clbA*.

The mean Ct values for *clbB*, *clbA*, and *clbQ* were 36.29, 34.81, and 34.16 in the benign neoplastic group, whereas they were 36.05, 31.82, and 33.0 in the malignant neoplastic group, respectively. Regarding nonneoplastic group, the mean Ct values for *clbB*, *clbA*, and *clbQ* were 34.27, 38.27, and 37.79, respectively, in the IBD group; 37.03, 37.73, and 38.96, respectively, in the inflammatory non-IBD group; and 33.06, 36.04, and 35.22, respectively, in the noninflammatory control group.

The comparison between the total number of patients in different studied groups according to the Ct values for *clbB* gene is shown in Table 1. Overall, there was a highly statistically significant difference between the total number of patients in different studied groups according to their Ct values (P<0.001). In addition, this table shows that there was a statistically significant difference between benign group and malignant group (P_1<0.001) and also neoplastic and nonneoplastic groups (P_3=0.005). In contrary, there was no significant difference between inflammatory and noninflammatory control groups.

In the present study, among 100 neoplastic patients, 42 (42%) malignant patients were positive for *pks*+*E. coli*, whereas 15 (15%) benign patients tested positive. However, among 50 nonneoplastic patients, in the inflammatory non-IBD group, there were seven (14%) positive, and in the IBD group, five (10%) patients were also positive for *pks E. coli*. On the contrary, *pks E. coli* was found to be positive in three (6%) of 20 noninflammatory control individuals.

The *pks*+*E. coli* was found to be significantly higher in biopsy samples of malignant patients (42%) than benign patients (15%) (P<0.001), in addition to a significant difference in the presence of the *pks* island between inflammatory group (85%) and noninflammatory control group (15%) (P<0.001), as shown in Fig. 1. There is also an overall significant difference between the prevalence of *pks* island in neoplastic patients (57%) compared with nonneoplastic patients (30%) (P<0.001), as shown in Table 2.

The *pks*+prevalence in neoplastic group was 57% and in the nonneoplastic inflammatory group was 40%, with no statistically significant difference between the two groups (P_1=0.102). However, the prevalence of *pks*+*E. coli* showed statistically significant difference among neoplastic group (57%) and their counterpart nonneoplastic noninflammatory control group (15%) (P_2=0.001), shown in Table 3.

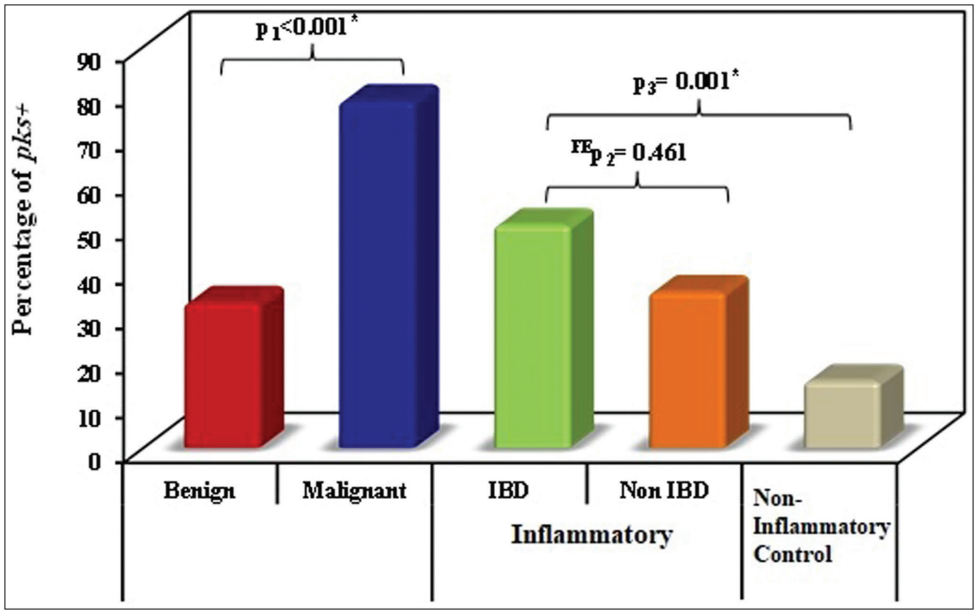
Discussion

CRC-associated *E. coli* strains are known to harbor the *pks* genomic island. This island is responsible for the synthesis of colibactin genotoxin [11]. The aim of the present study was to assess the possible link between pks+*E. coli* genotoxin and CRC. A total of 150 patients were recruited in this study, and then according to the pathological diagnosis, the neoplastic group (n=100) was subdivided into 46% benign and 54% malignant

Table 1 Comparison between the total number of patients in different studied groups according to the Ct values for *clbB* gene

	Neoplastic (N=100)		Nonneoplastic (N=50)				
			Inflammatory (N=30)		Noninflammatory control (N=20)		
Number of patients with cycle threshold value for *clbB*	Benign (N=46) [n (%)]	Malignant (N=54) [n (%)]	IBD (N=10) [n (%)]	Non-IBD (N=20) [n (%)]	n (%)	χ^2	^{MC}P
Low Ct value (<30 cycle)	3 (6.5)	5 (9.3)	1 (10.0)	2 (10.0)	1 (5.0)		
High Ct value (>30 cycle)	12 (26.1)	37 (68.5)	4 (40.0)	5 (25.0)	2 (10.0)	36.694*	<0.001*
Negative Ct value	31 (67.4)	12 (22.2)	5 (50.0)	13 (65.0)	17 (85.0)		
Significance between groups	$^{MC}P_1$<0.001*, $^{MC}P_2$=0.158, P_3=0.005*						

Figure 1



Comparison between the different studied groups according to presence of pks+Escherichia coli.

Table 2 Comparison between neoplastic and nonneoplastic groups according to presence of *pks+Escherichia coli*

	Neoplastic (*N*=100) [*n* (%)]	Nonneoplastic (*N*=50) [*n* (%)]	$<\sigma\upsilon\pi><\sigma\upsilon\pi>\chi^2$	*P*
Pks+	57 (57.0)	15 (30.0)	9.736*	0.002*

χ^2, χ^2 test. *P*: *P* value for comparing between the studied groups *Statistically significant at *P* value less than or equal to 0.05.

Table 3 Comparison of *pks+Escherichia coli* among each categorical group

	Neoplastic (*N*=100) [*n* (%)]	Inflammatory (*N*=30) [*n* (%)]	Noninflammatory control (*N*=20) [*n* (%)]	$<\sigma\upsilon\pi><\sigma\upsilon\pi>\chi^2$	*P*
pks+	57 (57.0)	12 (40.0)	3 (15.0)	12.740*	0.002*
Significance between groups		P_1=0.102, P_2=0.001*			

χ^2, χ^2 test. *P*: *P* value for comparing between the studied groups. P_1: *P* value for comparing between neoplastic and inflammatory. P_2: *P* value for comparing between neoplastic and noninflammatory control. *Statistically significant at *P* value less than or equal to 0.05.

patients. The nonneoplastic group (*n*=50) was subdivided into 40% noninflammatory control group and 40% inflammatory non-IBD patients, and 20% patients with IBD.

Our findings were comparable to other researches from different countries in which they included the same pathological groups. A study conducted in Hamadan University of Medical Science, Iran, obtained intestinal biopsy samples from 40 normal individuals, 40 patients with IBD, and 40 patients with CRC among 120 patients [12]. Similar to these results, a study preformed in United Kingdom, and University of Southampton, reported 21 patients with ulcerative colitis, 14 patients with Crohn's disease, 21 patients with colon cancer, and 24 controls [5].

Concerning the sex of the participants in this study, no statistically significant difference was observed among the study groups (*P*=0.059). Males dominated in the malignant neoplastic group, whereas females dominated in the nonmalignant neoplastic group. This result was in agreement with a Malaysian study that was carried out on 71 human patients. They found that 52 and 47.8% were males and females, respectively [5]. Shimpoh *et al.* [8] found that male prevalence was significantly greater among patients with CRC compared with the control group. Lack of a statistically significant difference regarding sex in our study may be attributed to limited number of patients and limited time frame.

Regarding patients' age, which ranged from 18 to 98 years old, there was a higher incidence of malignant neoplastic diseases in patients above 45 years old. The age of malignant patients was significantly higher than nonmalignant patients (*P*<0.001). In consistence with our results, a Malaysian study reported a higher rate of CRC disease in adults above 45 years [5].

16S rRNA gene was amplified in all biopsy samples to ensure the presence of bacterial DNA. No statistically significant difference in the mean Ct values among different pathological groups was found (P=0.906). Shimpoh *et al.* [8] and Burns *et al.* [13] used this universal bacterial sequence (16S rRNA) to detect total bacterial DNA as well.The genotoxic *E. coli* strains were identified as *pks*+*E. coli*, by amplifying *clbB*, *clbQ*, and *clbA* genes. All samples that were positive for *clbB* were also positive for both *clbQ a*nd *clbA*. Shimpoh *et al.* [8] reported the same results.

The *pks*+*E. coli* was found to be significantly higher in biopsy samples of malignant patients (42%) than benign patients (15%) (P<0.001), in addition to a significant difference in the presence of the *pks* island between inflammatory group (85%) and noninflammatory control group (15%) (P<0.001). There is also an overall statistically significant difference between the prevalence of *pks* island in neoplastic patients (57%) compared with nonneoplastic patients (30%) (P<0.001). The presence of *pks*+*E. coli* in patients with unremarkable findings may be attributed to missed carcinogenic and/or inflammatory lesions during conventional colonoscopy [14–16].

This study agrees with earlier researches, wherein *pks*+*E. coli* was over-represented in the tissue biopsy samples of patients with CRC as compared with those without cancer [17–19].

In disagreement with these findings, a Japanese study revealed that the *pks* prevalence was 43 and 51% in CRC and adenoma cases, respectively. However, it was 46% among the controls [8]. These differences may be attributed to other overlapping factors that may predispose to CRC such as, diet, life style, geographic area, and antibiotic abuse.

Although the findings of this study strongly support the suggestion that *pks*+*E. coli* plays a crucial role in the pathogenesis of CRC, more research studies are needed to pave the way for a stronger scientific evidence on the role of the microbiota in CRC development [20].

Conflicts of interest

There are no conflicts of interest.

References

1 Faïs T, Delmas J, Cougnoux A, Dalmasso G, Bonnet R. Targeting colorectal cancer-associated bacteria: a new area of research for personalized treatments. Gut Microbes 2016; 7:329–333.

2 Keum N, Giovannucci E. Global burden of colorectal cancer: emerging trends, risk factors and prevention strategies. Nat Rev Gastroenterol Hepatol 2019; 16:713–732.

3 Bonnet M, Buc E, Sauvanet P, Darcha C, Dubois D, Pereira B, *et al*. Colonization of the human gut by *E. coli* and colorectal cancer risk colonization of human gut by *E. coli* and colorectal cancer. Clin Cancer Res 2014; 20:859–867.

4 Bossuet-Greif N, Vignard J, Taieb F, Mirey G, Dubois D, Petit C, Oswald E, Nougayrède JP. The colibactin genotoxin generates DNA interstrand cross-links in infected cells. mBio 2018; 9:e02393–17.

5 Iyadorai T, Mariappan V, Vellasamy KM, Wanyiri JW, Roslani AC, Lee GK, *et al*. Prevalence and association of pks+ *Escherichia coli* with colorectal cancer in patients at the University Malaya Medical Centre, Malaysia. PLoS ONE 2020; 15:e0228217.

6 Li ZR, Li J, Cai W, Lai JY, McKinnie SM, Zhang WP, *et al*. Macrocyclic colibactin induces DNA double-strand breaks via copper-mediated oxidative cleavage. Nat Chem 2019; 11:880–889.

7 Wilson MR, Jiang Y, Villalta PW, Stornetta A, Boudreau PD, Carrá A, *et al*. The human gut bacterial genotoxin colibactin alkylates DNA. Science 2019; 363:eaar7785.

8 Shimpoh T, Hirata Y, Ihara S, Suzuki N, Kinoshita H, Hayakawa Y, *et al*. Prevalence of pks-positive *Escherichia coli* in Japanese patients with or without colorectal cancer. Gut Pathog 2017; 9:1–8.

9 Johnson JR, Johnston B, Kuskowski MA, Nougayrede JP, Oswald E. Molecular epidemiology and phylogenetic distribution of the *Escherichia coli* pks genomic island. J Clin Microbiol 2008; 46:3906–3911.

10 Tang-Fichaux M, Branchu P, Nougayrède JP, Oswald E. Tackling the threat of cancer due to pathobionts producing colibactin: is mesalamine the magic bullet?. Toxins 2021; 13:897.

11 Faïs T, Delmas J, Barnich N, Bonnet R, Dalmasso G. Colibactin: more than a new bacterial toxin. Toxins 2018; 10:151.

12 Zarei O, Arabestan MR, Majlesi A, Mohammadi Y, Alikhani MY. Determination of virulence determinants of *Escherichia coli* strains isolated from patients with colorectal cancer compared to the healthy subjects. Gastroenterol Hepatol Bed Bench 2019; 12:52.

13 Burns MB, Lynch J, Starr TK, Knights D, Blekhman R. Virulence genes are a signature of the microbiome in the colorectal tumor microenvironment. Genome medicine. 2015;7:1–2.

14 Lee J, Park SW, Kim YS, Lee KJ, Sung H, Song PH, Yoon WJ, Moon JS. Risk factors of missed colorectal lesions after colonoscopy. Medicine 2017; 96:27.

15 Park SY, Moon W, Park SJ, Park MI, Kim KJ, Kim SJ, *et al*. The colonoscopic miss rates of colorectal polyps as determined by a polypectomy. Korean J Gastrointest Endosc 2008; 36:132–137.

16 Lou GC, Yang JM, Xu QS, Huang W, Shi SG. A retrospective study on endoscopic missing diagnosis of colorectal polyp and its related factors. Turk J Gastroenterol 2014; 25(Suppl 1):182–186.

17 Raisch J, Buc E, Bonnet M, Sauvanet P, Vazeille E, De Vallée A, *et al*. Colon cancer-associated B2 *Escherichia coli* colonize gut mucosa and promote cell proliferation. World J Gastroenterol 2014; 20:6560.

18 Xie LL, Wu N, Zhu YM, Qiu XY, Chen GD, Zhang LM, Liu YL. Expression of enterotoxigenic *Bacteroides fragilis* and polyketide synthase gene-expressing *Escherichia coli* in colorectal adenoma patients. Zhonghua Yi Xue Za Zhi 2016; 96:954–959.

19 Feng Y, Mannion A, Madden CM, Swennes AG, Townes C, Byrd C, *et al*. Cytotoxic *Escherichia coli* strains encoding colibactin and cytotoxic necrotizing factor (CNF) colonize laboratory macaques. Gut Pathog 2017; 9:1–5.

20 Buc E, Dubois D, Sauvanet P, Raisch J, Delmas J, Darfeuille-Michaud A, *et al*. High prevalence of mucosa-associated *E. coli* producing cyclomodulin and genotoxin in colon cancer. PLoS ONE 2013; 8:e56964.