



## Prevalence and association of *pks*+ *Escherichia coli* with colorectal cancer in Iraqi patients

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### Abstract

Colorectal cancer (CRC) is a malignant tumor in the digestive system whose incidence and mortality are high-ranking among tumors worldwide. The present work aimed to investigate the role of colibactin-produced isolates of *pks*+ *Escherichia coli* in CRC disease. Among 159 specimens, which were subjected to different examinations, a total of 57.36% (n=88) of *E. coli* isolates were obtained, including 32.08% (n=51) of isolates from patients with CRC, including 21.4% (n=34) from fecal specimens and 10.7% (n=17) from tissue specimens as well as 23.3% (n=37) of isolates were obtained from healthy controls, including 16.4% (n=26) from fecal specimens and 6.92% (n=11) from tissue specimens. Genomic DNA was extracted from 57 selected *E. coli* isolates, which obtained 20 specimens (10 from CRC patients and 10 from controls) and 37 fecal specimens (21 from CRC patients and 16 from controls) for molecular detections of two genes (*uidA* and *pks*). All isolates of *E. coli* harbored *uidA* gene, while 11 (55.0%) of isolates were given positive for *pks*, including 8 (80.0%) from CRC patients and 3 (30.0%) from healthy controls. Immunofluorescence assay (IFA) was utilized to study the effect of *pks*<sup>+</sup> and *pks*<sup>-</sup> *E. coli* isolates on Normal human derived adipose tissue (NHF) cell line for determining the quantification of *H2AX* expression. The results indicated that there was significant variation in *H2AX* expression between *pks*<sup>+</sup> *E. coli* isolate-treated NHF and other groups. However, there was a non-significantly variation in *H2AX* expression between *pks*<sup>-</sup> *E. coli* isolate treated NHF and untreated NHF (as control) tissues. The results indicated that high effect of *pks*<sup>+</sup> *E. coli* on *H2AX* expression in NHF cell line.

**Keywords:** *pks*, *uidA*, Colorectal Cancer, *H2AX* Expression.

### Introduction

Colorectal cancer (CRC) is one of the most common cancers worldwide, with between one and two million new cases being diagnosed every year, thus making CRC the third most common cancer and the fourth most common cause of death related to tumors, with seven thousand deaths annually, exceeded only by stomach, liver and lung cancers. CRC ranks second among female cancers (9.2%), third among male cancers (10%) (Mármol et al., 2017). It is influenced by many risk factors, including; heredity, lifestyle, gut microbiota, diet, obesity, and other factors (Sun and Kato, 2016).

The intestinal microbiota, composed of a large population of microorganisms, is often considered a “forgotten organ” in human health and diseases (Ling et al., 2022). Increasing evidence indicates that intestinal microbiota dysbiosis is closely related to colorectal cancer (CRC). It is becoming obvious that gut microbes played crucial roles in both the initiation and progression of CRC. Recent studies have identified *Peptostreptococcus anaerobius*, *E. coli*, *Enterococcus faecalis*, *Fusobacterium nucleatum*, *Bacteroides fragilis* and *Streptococcus bovis*, as candidate pathogens of CRC (Cheng et al., 2020).

Microbiome dysbiosis is increasingly being associated to disease pathogenesis, and several different types of bacteria, including genotoxic and enterotoxigenic *E. coli*, have been studied as possible contributors to colorectal cancer (Butt et al., 2021; Gholizadeh et al., 2019). Enterotoxigenic *E. coli* (ETEC) is a pathogenic type of *E. coli* that is found in every part of the world with limited access to healthcare and food (Fleckenstein and Kuhlmann, 2019). The members of the gut microbiota have a lifelong impact on the health and sickness of the mammals that host them. Despite the importance of this microbiome to normal growth and immunity, disruptions in this ecosystem have been related to metabolic syndrome, inflammatory bowel disease, cancer, and other chronic conditions. The overgrowth or mislocalization of potentially dangerous elements of the gut microbiota, such as *pks+* *E. coli*, which generates the genotoxin colibactin, has been associated to an increased risk of cancer, including colorectal cancer (CRC) (Gaab et al., 2023). *E. coli*, that contain *pks* (polyketide synthase island) can be found in the gut microbiota of both CRC patients and healthy people. The genotoxin colibactin expression is controlled by genes located on this genomic island that code for components of the biosynthesis pathway of a non-ribosomal peptide hybrid polyketide. Chromosomal abnormalities, senescence and stoppage of the cell cycle are the results of DNA double-strand breaks (DSBs) induced by colibactin in mammalian cells (Oliero et al., 2021).

In this context, the present work was aimed to investigate role of colibactin-produced isolates of *pks+* *E. coli* in CRC disease.

## Materials and Methods

### Collection of specimens

Fifty-six tissue specimens, including 31 colorectal tissue specimens and 25 healthy normal tissue specimens were collected from individuals, of different ages (ranging between 26 and 85 years) and sexes (males and females), were attained at different hospitals in Baghdad city during the period of March 2021 and September 2022. The age of CRC patients was ranged between 38 and 85 years and the age of health controls was ranged between 26 and 85 years.

### Isolation and identification of bacterial isolates

All samples were transferred by sterile swab transporter media into the laboratory and each swab was streaked on MacConkey plates. The plates were placed in an incubator at 37°C for 24 hours. The growing isolates were recultured on nutrient agar plates and subjected into different examinations, microscopic (gram staining), cultural characteristics (on different media, including Eosin methylene blue EMB and blood agar plates) and biochemical tests (vogas-Proskauer, urease, oxidase, methyl red, indole and catalase) (Lihan et al., 2021; Mahmood and Manaf, 2017).

## Molecular detection of *uidA* and *pks*

### DNA extraction

For DNA extraction, the Presto™ Mini gDNA Bacteria Kit (INTRON\Korea) was utilized.

### Primers utilized in this study

Primers listed in the Table 1 were supplied by macrogen® (Korea) company for detection of *uidA* and *pks* genes of *E. coli*.

**Table 1. The name, sequence and product size of primers utilized in this study**

Gene		Primer Sequence (5'-3')	Size (bp)
<i>pks</i>	F	TCGATATAGTCACGCCACCA	237
<i>pks</i>	R	GTCAAGCGAGCATACGAACA	
<i>uidA</i>	F	CGTCAATGTAATGTTCTGCGA	333
<i>uidA</i>	R	AAATCGGCGAAATTCCATACC	

F: Forward, R: Reverse.

### Detection of *uidA* and *pks* genes by Polymerase Chain Reaction

PCR was utilized to screen the isolates for the following genes: *uidA* and *pks*. DNA amplification from bacteria was accomplished in a total volume of 25 µl containing 12.5 µl of OneTaq® 2X Master Mix green, 5 µl (100 of DNA, 4.5 µl nuclease free water, and 1.5 µl of each primer. The PCR was done with a Gradient Thermocycler under the following conditions: denaturation for 1 min at 95°C; 30 cycles of 30 s at 95°C, 30 s at 57°C, and 1 min at 72°C; and a final extension step for 5 min at 72°C. A 10 ml aliquot of the PCR product experienced gel electrophoresis on 2% agarose, followed by staining with red-safe solution. The fluorescence, induced by UV, was utilized to reveal amplified fragments of specific sizes of DNA, and the amplicons' size was estimated by comparing them to a ladder of DNA consisting of 1000 bp that was also included on the same gel (Mahmood, 2022).

### *In vitro* cytotoxic activity of *pks+* and *pks-* *E. coli* isolates Encoding) on normal human fibroblast (NHF) cell line

Immunofluorescent assay (IF) was utilized to investigate the effect of *E. coli* bacteria holding *pks+* gene on Normal Human Fibroblast (NHF) Cell Line and compare the results with *E. coli* negative for this gene. A normal human-derived adipose tissue (NHF) cell line was utilized (Safi et al., 2019). NHF cell line, were maintained in 10% fetal bovine supplemented-MEM containing 100 µg/mL streptomycin and 100 units/mL penicillin. Trypsin-EDTA reseeding at

50% confluence twice weekly during 37°C incubation was utilized for cell passage (Mammedova et al., 2023). The cells number in every four squares of heamacytometer was counted, after that, the average was counted and multiplied by 104 to fixed the cells number in 1 ml (1000 µl). About 0.2 ml of cell suspensions (sample of cells calculated) through an Improved Double Neubauer Ruling Counting Chamber (Assistant, Germany), 400X magnification power was utilized to calculate the cells (Ongena et al., 2010). Viable cells seem to shine, while dead cells seem dark in the light microscope. the cells number for each unit volume (cells/ml) was calculated using the following formula:

$$c = n * d * 104$$

c = the viable cells number for each milliliter, n= the counted viable cells number, d= 10, which is factor of dilution (Freshney, 2015).

NHF cells were seeded at 5,000 cells/well, at the next day NHF cells were exposed to *pks+* and *pks-* at a moi 20 and un-treated (control). Next 48hr. of exposure, the viability of

gene cells has been assessed using IF assay for determining the quantification of H2AX expression (Cuevas-Ramos et al., 2010). The cells were exposed to a standard step ladder of rinsing (PBS for 3 times), then cell fixation by using 4% paraformaldehyde, 30 min, at RT), permeabilization using 0.5% Triton-X, 30 min, at RT), and blocking using 10% goat serum, 60 min). The cells were treated with 1 µgmL<sup>-1</sup> of each of the primary markers, in an incubator for overnight at 4 °C. Then, the cells were treated with 1 µgmL<sup>-1</sup> of the secondary antibodies (Albino et al., 2010), and cells were examined using a glowing fluorescent microscope (as manufactory recommendation).

## Results and Discussion

### Isolation and Identification of bacterial isolates

All samples were subjected to various examinations, including microscopic examination, cultural characteristics on media, biochemical tests, in order to isolate and identify bacterial isolates of *E. coli*. The results of examinations were illustrated as the following Table 2.

**Table 2. Results of identification of E. coli isolates from different samples**

Property	Test	Result
<b>Microscopic</b>	Gram staining	Negative
	Shape (Cocci/ Diplococci/ Rods)	Rod
	Capsule	Present
	Spore	Absent
	Motility	Motile (peritrichous flagella) or non-motile
<b>Cultural</b>	Hemolysis (Alfa/ Beta/ Gamma)	Beta-hemolysis
<b>Characteristics</b>	MacConkey agar	Pink colonies
	EMB agar	green metallic sheen colonies
<b>Biochemical</b>	Vogas-Proskauer	Negative
	Urease	Negative
	Oxidase	Negative
	Methyl red	Positive
	Indole	Positive
	Catalase	Positive

Among 159 specimens, which were subjected to previously mentioned examinations, a total of 57.36% (n=88) of *E. coli* isolates were obtained, including 32.08% (n=51) of isolates from patients with CRC, including 21.4% (n=34) from fecal

specimens and 10.7% (n=17) from tissue specimens as well as 23.3% (n=37) of isolates were obtained from healthy controls, including 16.4% (n=26) from fecal specimens and 6.92% (n=11) from tissue specimens, as shown in Table 3.

**Table 3. The distribution of E. coli isolates according to source**

Source	Patients		Controls	
	N	%	N	%
Fecal specimens	n=34	21.4	n=26	16.4
Tissue specimens	n=17	10.7	n=11	6.92
Total (n=88; 57.36%)	n=51	32.08	n=37	23.3
Total Specimens (n=159; 100%)				

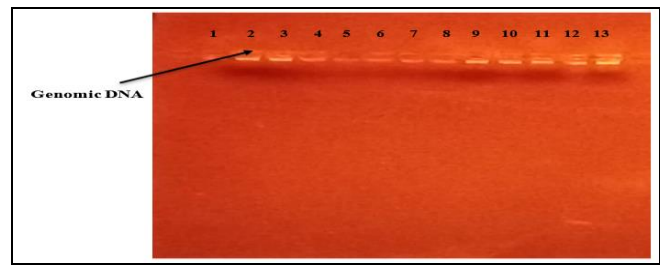
Correlations between the composition of the gut microbiota and CRC put it in third place among malignancies (Alexander et al., 2018). Cancer patients who have dysbiosis of their gut microbiota are more likely to develop tumours and spread their disease (Han et al., 2018). Strains of *E. coli* obtained from the microbiota of colorectal cancer patients' guts have recently been revealed to play a role in carcinogenesis (Eklöf et al., 2018). Chronic inflammation, which can result from infection of pathogenic bacteria by its impact on the microbiota and the host, has been associated to *E. coli*'s role in the promotion and progression of CRC (Zarei et al., 2019). Furthermore, the faecal microbiome exhibits changes in the earliest stages of CRC development, suggesting that it may contribute to the disease (Yachida et al., 2019).

Based on the findings of this work, the *E. coli* existed to be more prevalent in patients with CRC as compared to normal healthy individuals, in agreement with Iyadorai et al., (2020) who found that the majority of *E. coli* isolates (14.7%) were obtained from patients with CRC compared with controls (4.3%). Different studies have described that between 82% and 71% of colonic carcinoma patients are highly *E. coli*-colonized compared to health individuals (Zarei et al., 2019). Fazeli and Hasan, (2021) reported that the relative abundance of *E. coli* isolates was significantly greater in patients of CRC (14/30; 46.6%) than in healthy individuals (5/30; 16.6%). Magdy et al. (2015) have earlier envisaged the *E. coli* isolates presence in about 50% of specimens of CRC and 20% healthy individuals. However, Nouri et al. (2021) reported that the investigation of the *E. coli* isolates presence showed that the specimens without *E. coli* was considerably higher in specimens of control ( $n = 10/50$ ; 20%) than in those with patients with CRC ( $n = 2/50$ ; 4%),  $P = 0.028$ .

### Molecular Detection of *uidA* and *pks* genes

#### DNA Extraction

Genomic DNA was extracted from 57 selected *E. coli* isolates, which obtained 20 specimens (10 from CRC patients and 10 from controls) and 37 fecal specimens (21 from CRC patients and 16 from controls) for molecular detections of three genes, including *pks* and *uidA*. Extraction of DNA was achieved by using bacterial DNA extraction kit (Geneaid\Korea), the kit afford a good result for DNA concentration and purity. Nano drop Spectrophotometer was then utilized to assess DNA concentrations and Purity. The purity ranged from (1.7-2.0) and the concentration (50-130) ng/μl, while the DNA safety was detected by (agarose gel electrophoresis) as a single clear band with no smears when visualized under UV light, as shown in Fig 1.

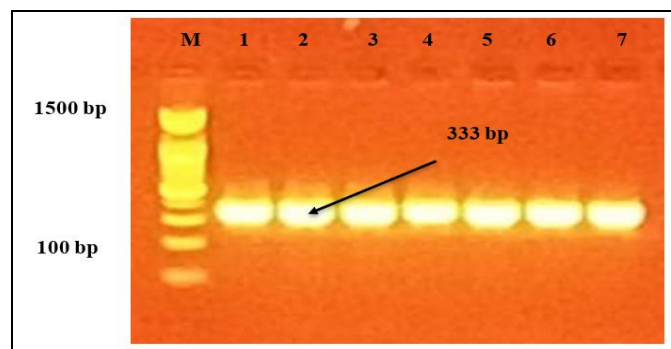


**Fig. 1** Genomic DNA extracted from *E. coli* isolates after electrophoresis on agarose 1% stained with ethidium bromide, carried out for 90 minutes at 90 volts visualized under UV transilluminator

#### Molecular Detection of *pks* and *Uida*

Molecular detection of three genes (*pks* and *Uida*) were investigated in the examined isolates using PCR with specific primer, as described below.

All isolates of *E. coli* were harbored *uidA* gene, as shown in Fig 2. The PCR product appeared as single DNA band with a molecular base of 333 bp on gel electrophoresis.



**Fig. 2** Gel electrophoresis in agarose for *uidA* gene amplification (333 bp) using PCR among isolates of *E. coli* in (2% agarose), for 90 minutes under 70 volts. M: ladder of DNA, lanes (1-7): positive result for gene.

The  $\beta$ -D-glucuronidase, encoded by the *uidA* gene, is commonly utilized to specifically identify *E. coli* (Molina et al., 2015). Adekanmbi et al. (2020) reported that twenty-four *E. coli* possessing the *uidA* gene were obtained from a pool of thirty-eight *E. coli* obtained. Using *uidA* gene, only 50 isolates (50/76, 65.8%) were confirmed as *E. coli* (Zarei et al., 2023). Also, the number of malignant tumors (44/62; 71%) positive for the *uidA* gene was not significantly different ( $p=0.3428$ ) from benign (38/62; 61%) tumors (Villariba-Tolentino et al., 2021). Among 20 isolates of *E. coli*, 11 (55.0%) of isolates were given positive for *pks*, including 8 (80.0%) from CRC patients and 3 (30.0%) from healthy controls, as shown in Table 4. The PCR product appeared as single DNA band with a molecular base of 237 bp on gel electrophoresis, as shown in Fig 3.

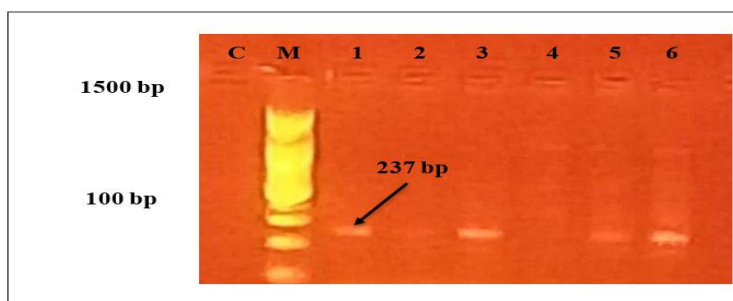


Fig. 3 Gel electrophoresis in agarose for *pks* gene amplification (237 bp) using PCR among isolates of *E. coli* in (2% agarose), for 90 minutes under 70 volts. M: ladder of DNA, C: negative control, lanes (1, 3, 5 and 6): positive result for gene, lanes (2 and 4): negative result for gene.

Table 4. The distribution of *pks* among isolates of *E. coli* isolated from tissue specimens

<i>pks</i> gene status	Studied groups (N,%)		Total	P-value
	Patient	Control		
Positive	8 (80.0%)	3 (30.0%)	11 (55.0%)	0.02 Significant
Negative	2 (20.0%)	7 (70.0%)	9 (45.0%)	
Total	10 (100.0%)	10 (100.0%)	20 (100.0%)	

Among 37 isolates of *E. coli* obtained from fecal specimens, 48.6% (n=18) of isolates were harbored *pks* gene, including

61.9% (n=13) from CRC patients and 31.3% (n=5) from controls, as shown in Table 5.

Table 2. The distribution of *pks* among isolates of *E. coli* isolated from fecal specimens

<i>pks</i> gene status	Studied groups (N,%)		Total	P-value
	Patient	Control		
Negative	8 (38.1%)	11 (68.8%)	19 (51.4%)	0.06 N.S
Positive	13 (61.9%)	5 (31.3%)	18 (48.6%)	
Total	21 (100.0%)	16 (100.0%)	37 (100.0%)	

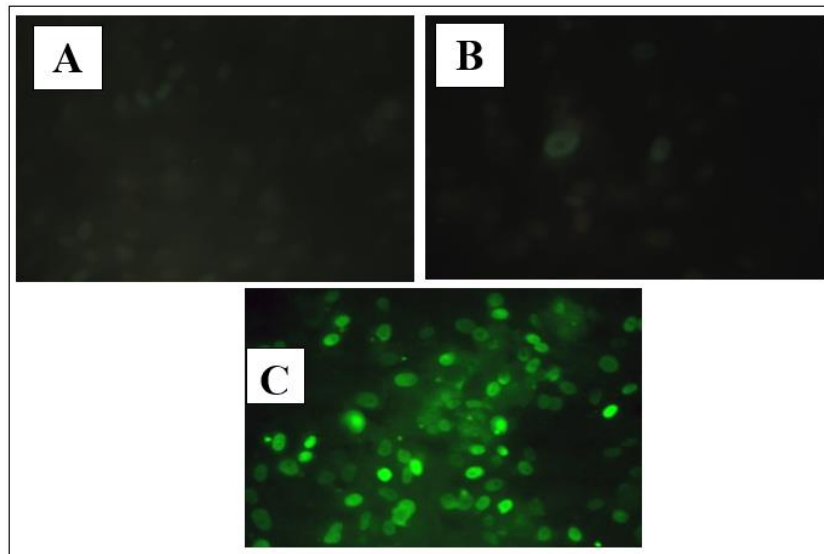
The results indicated that there were significant differences in presence of *pks* among *E. coli* isolates from tissue specimens in patients and controls, but not for fecal specimens, in agreement with Shimpoh et al., (2017), which may attribute to that the tested fecal specimens were few specimens, or may feces be aggregated from different parts of gastrointestinal tract, not only from colon (Hua, 2020).

Among 139 *E. coli* isolates were studied, the *pks* gene was detected in 56 isolates (40%) in study conducted by McCoy et al. (2021). Fabian et al. (2020) reported that twelve of the 26 isolates (46.2 %) were PCR-positive for the *pks* genes. Also, Of the 69 isolates, 45 (65%) were positive for *pks* in study conducted by Nouri et al. (2021). Results reported by Iyadorai et al. (2020) showed 16.7% and 4.3% of CRC and healthy controls, respectively were *pks*<sup>+</sup> *E. coli*.

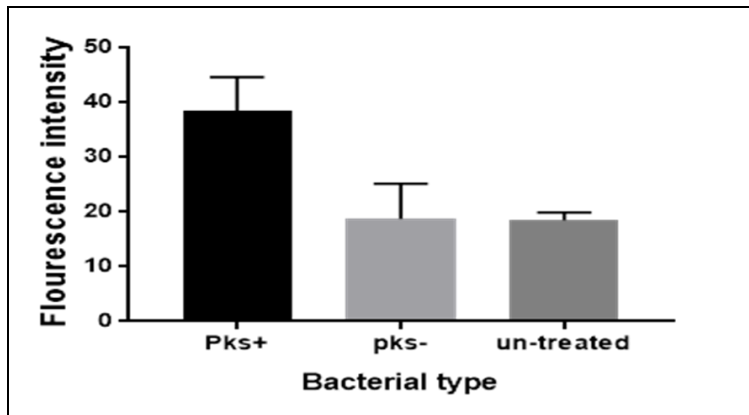
**Effect of *pks*<sup>+</sup> and *pks*<sup>-</sup> *E. coli* isolates on Normal human derived adipose tissue (NHF) cell line**

Immunofluorescence assay (IFA) was utilized to study the effect of *pks*<sup>+</sup> and *pks*<sup>-</sup> *E. coli* isolates on Normal human derived adipose tissue (NHF) cell line for determining the quantification of *H2AX* expression. Three specimens of NHF was utilized as follows: *pks*<sup>+</sup> *E. coli* isolate treated NHF, *pks*<sup>-</sup> *E. coli* isolate treated NHF and untreated NHF (as control) tissues. The results were shown in Fig. 4 and Fig 5.

The results indicated that there was significant difference in *H2AX* expression between *pks*<sup>+</sup> *E. coli* isolate treated NHF and other groups. However, there was non-significant difference in *H2AX* expression between *pks*<sup>-</sup> *E. coli* isolate treated NHF and untreated NHF (as control) tissues. The results were indicated that highly effect of *pks*<sup>+</sup> *E. coli* on *H2AX* expression in NHF cell line.



**Fig. 4** Immunofluorescent images for (A: untreated NHF as control; B: *pks<sup>-</sup>* *E. coli* isolate treated NHF and C: *pks<sup>+</sup>* *E. coli* isolate treated NHF) after 48 hours from infection



**Fig. 5** Immunofluorescent intensity for untreated NHF as control; *pks<sup>-</sup>* *E. coli* isolate treated NHF and *pks<sup>+</sup>* *E. coli* isolate treated NHF after 48 hours from infection

Subsequent studies utilizing *in vitro* models established a distinct mutational signature after exposure to *pks<sup>+</sup>* *E. coli*, and these signatures were identified in roughly 5% of colorectal cancer patients, consistent with the hypothesised nucleotide-binding activity of the recently proposed colibactin structure (Dougherty and Jobin, 2021).

Correspondingly, Cuevas-Ramos et al. (2010) found that low-dose exposure of cultured mammalian epithelial cells to live *pks<sup>+</sup>* *E. coli* induced a transient DNA damage response, followed by cell division with signs of incomplete DNA repair, resulting in anaphase bridges and chromosome aberrations. Up to 21 days post-infection, dividing cells showed persistent micronuclei, aneuploidy, ring chromosomes, and anaphase bridges, all of which are indicative of breakage-fusion-bridge cycles and chromosomal instability. The mutagenic and transformative potential of the infection was shown by an increase in the frequency of gene

mutations and the establishment of anchorage-independent colonies in exposed cells. As a result, colonization with these *E. coli* strains harbouring the *pks* island may lead to the development of sporadic colorectal cancer (Dougherty and Jobin, 2021).

The mutational signature left by other oncogenic bacteria, such as *pks<sup>+</sup>* *E. coli*, is seen in colon 334 carcinoma, as was recently observed (Traulsen et al., 2021). According to the gnotobiotic mouse model, *pks<sup>+</sup>* *E. coli* does not directly interact with the epithelium or damage colonic integrity under homeostatic settings, as observed by Harnack et al., (2023). Mice colonized with an isogenic *clbR* mutant unable to produce colibactin rapidly recover from mucosal disruption, while *pks<sup>+</sup>* *E. coli* obtain direct access to the epithelium and cause epithelial damage and persistent colitis. Mice colonised with *pks<sup>+</sup>* *E. coli* cannot recover their ability to form a protective barrier. To continue the cycle, *pks<sup>+</sup>* *E.*

*coli* adheres to the epithelium and causes chronic mucosal inflammation with morphological and transcriptional features similar to human ulcerative colitis.

Separation of mammalian cells and bacteria by cell-impermeable membranes attenuates colibactin's cytotoxic effects, demonstrating that cell-to-cell contact is required for genotoxicity after *pks+* infection in vitro. It is unknown whether or not pathogenic bacteria require specialized secretion systems in order to transport colibactin to host cells, however, the compound's instability may prevent remote delivery. There have been no discovered transporters that are crucial for *pks+* genotoxicity. Interestingly, previous investigations showing that preventing bacteria from internalizing BAC*pks* had no effect on their genotoxicity were conducted (Dougherty and Jobin, 2021). In contrast, it was shown that a clinical *pks+* AIEC isolate from a CRC patient may penetrate and reproduce within epithelial cells, leading to a rise in tumor growth (Lucas et al., 2020). Because *pks+* *E. coli* enhances tumor formation in pre-clinical settings and genetic abnormalities are characteristic of nearly all malignancies (Dougherty and Jobin, 2021).

This study indicated the prevalence of *pks* was high in isolates of *E. coli* obtained from CRC. Also, *pks* gene may have a critical role in the incidence of CRC.

## Conclusion

In conclusion, this study sheds light on the potential role of colibactin-producing isolates of *pks+* *Escherichia coli* in colorectal cancer (CRC) disease. The findings revealed a relatively high prevalence of *pks+* *E. coli* isolates, particularly in CRC patients, suggesting their potential involvement in the development or progression of CRC. Molecular analysis demonstrated that all *E. coli* isolates harbored the *uidA* gene, while a significant proportion, predominantly from CRC patients, were positive for the *pks* gene. Furthermore, functional experiments using the NHF cell line demonstrated a substantial impact of *pks+* *E. coli* isolates on H2AX expression, indicating their potential genotoxic effect. While further research is needed to elucidate the exact mechanisms and pathways through which *pks+* *E. coli* may contribute to CRC, these findings highlight the importance of understanding the role of specific bacterial strains in the complex landscape of colorectal cancer, potentially paving the way for targeted preventive and therapeutic strategies.

## Conflict of Interest

The author hereby declares no conflict of interest.

## Consent for publication

The author declares that the work has consent for publication.

## Ethical Considerations

The study was approved by the institutional ethical committee.

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