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Molecular mechanisms by which ClbA, ClbB and ClbQ genes component of colibactin contribute to *Escherichia coli*-induced colorectal tumorigenesis

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ABSTRACT

Background. *The importance of Escherichia coli bacteria is highlighted by being a pathogen and encoding genes that produce toxins that contribute to the occurrence of tumors in colon and rectal cancer.*

Aim of study. *Molecular detection of the most important genes produced by Escherichia coli bacteria and their role in causing colon and rectal tumors.*

Methods. *80 different samples were collected from patients with colon and rectal cancer and were clinically examined in the endoscopy unit inside Al-Sadr Teaching Hospital in Basra Governorate during the period from March 2022 to December 2023.*

Results. *Escherichia coli bacteria were isolated and diagnosed and were molecularly diagnosed by 16S rRNA sequencing. As for the genes ClbA, ClbB, and ClbQ, they were diagnosed using special primers and real-time PCR technology. CT values showed gene expression for all genes at high levels among infected individuals and lower than in the group of healthy individuals and were not detected in the second group of healthy individuals.*

Conclusion. *The results revealed a frequency of Escherichia. coli in infected individuals, accompanied by an increased expression of the virulence genes CblA, ClbB and ClbQ that encode colibactin. Real-time PCR analysis confirmed these results.*

Keywords: *Escherichia coli,* ClbA, ClbB, and ClbQ genes, colorectal cancer

INTRODUCTION

The most prevalent gastrointestinal cancer, colorectal cancer (CRC), is a significant worldwide health issue. After lung and breast cancer, colorectal cancer is the third most frequent cancer diagnosed globally, CRC is a common disease diagnosed in worldwide each year, where only in 2020 claiming nearly one million lives [1-3]. CRC isn't a single story, but rather a collection of narratives driven by diverse genetic changes. At the heart of it all lie mutations in colon stem cells, affecting genes like WNT, EGFR, TP53, and TGF-β that control growth and repair. These mutations, acquired over years, gradually transform healthy cells into cancerous ones. 1014 microbes colonize the muscularis mucosae, lamia propria, and epithelial cells that make up the human gut

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mucosa. These microorganisms are five times more numerous than human cells. An essential part of the human stomach's microbial ecosystem are gut bacteria. About 1000 types of commensal bacteria, the majority of which are unknown species of anaerobic strains, populate the stomach soon after birth [4].

Armed with a vast diversity of genes, microorganisms inhabit nearly every area of the human body and have the ability to interact, modify, or disrupt a wide range of human genes, particularly in colonic cells. It's interesting to note that the human microbiota encodes around 100 times as many proteins as the human genome. Between 1000 to 1500 bacterial species make up this microbiota, and each person's microbiome composition varies greatly [5]. *E. coli* is a broad category of rod-shaped, facultatively anaerobic, Gram-negative bacteria belonging to the Enterobacteriaceae family.

It is a part of the natural flora in the human gut and is oxidase-negative. It develops on simple carbon sources like glucose and acetate. Nevertheless, certain strains are harmful [6,7]. Enteropathogenic *E. coli* (EPEC), enterohemorrhagic *E. coli* (EHEC), Shiga-toxin-producing *E. coli* (STEC), enteroaggregative *E. coli* (EAEC), enterotoxigenic *E. coli* (ETEC), and diffusely adherent *E. coli* (DAEC) are the six pathotypes of intestinal pathogenic *E. coli* that are traditionally separated based on their pathogenicity profiles [8]. Based on phylogenetic study, *E. coli* strains are categorized into the following primary phylogenetic groups: A, B1, B2, and D. The majority of pathogenic strains are classified as B2 and D, whereas the majority of commensal strains are classified as A [9].

Phylogenetic analysis is used to classify E. coli strains into the main phylogenetic groupings (A, B1, B2, and D). Phylotype A comprises the majority of commensal strains, whereas phylotypes B2 and D comprise the majority of harmful strains. Such as genotoxins, which is substances or substances that have the potential to harm DNA or chromosomes, leading to mutations [10]. Colibactin is genotoxin a mix of hybrid compounds that include a polyketide and a peptide, which is encoding by pks island is A genomic island that is 54 kb long and contains at least 19 genes, which this The ClbA-S gene cluster contains polyketide synthases and nonribosomal peptides along with additional auxiliary enzymes [11].

Colibactin is detected by a group of genes involved in the regulation and biosynthesis of colibactin, which includes As a phosphopantetheinyl transferase-encoded gene, ClbA is a significant gene cluster in colibactin synthesis. It is primarily necessary for the production and maturation of colibactin and is typically linked to pathogenicity and cancer [12]. While the functional role of the ClbQ gene has not yet been discovered, it is believed that it plays a regulating function in the procedure of colibactin production, while the ClbB gene plays a role in the process of regulating colibactin synthesis. Colibactin which synthesized by bacteria colonizing the human body in the form non-toxic precursor colibactin begins as a harmless molecule called 19-precolibactin inside bacteria.

This precursor is then transformed into the active and toxic colibactin. An enzyme called ClbP plays a key role in this activation by removing a specific chemical group (Ndeacylation). Active and toxic colibactin has a unique structure it electrophilic cyclopropane group, which cause damage by alkylation DNA This involves attaching chemical group Alkyl group directly to DNA bases like adenine, leading to disruptions in cellular processes such Cell cycle stop and double-strand DNA breakage and megalocytosis, where Colibactin-induced Mutations are linked to DNA damage and chromosomal instability, and this lesions often seen in tumer [13-16]. Colibactin also affects the expression of uncontrolled division and tumor growth are made possible by mutations in DNA mismatch repair (MMR) proteins that impair or interfere with their activity [17,18].

This study aim of Molecular detection of the most important genes produced by Escherichia coli bacteria and their role in causing colon and rectal tumors.

MATERIALS AND METHODS

Study of group

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

Show all of these individuals symptoms and indicators including modifications to bowel motions, bloating and cramping weariness, unexplained weight loss, bleeding in the stool, and stomach pain breathing difficulties [19]. Direct interviews were used with each patient completing a questionnaire that included: name, age, gender, address and smoking. It covers questions related to epidemiological and clinical features and laboratory investigations of patients.

Inclusion criteria

Individuals who have been diagnosed with colorectal cancer and who fulfill the diagnostic requirements.

Exclusion criteria

Individuals with intestinal infections at the moment. Those who, in the two months before to enrollment, had used antibiotics or microecological agents. Patients who received adjuvant chemoradiotherapy before sampling. People with long-term conditions like diabetes, heart disease, and high blood pressure. Those who refuse to participate in our study.

Blood specimens

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

Stool specimens for culture

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

Stool specimens for gen expression

The stool was collected in a sterile a container with a dark color and an airtight lid, and a certain amount of stool was placed inside the container and transferred as soon as possible to the refrigerator and kept at a temperature of four degrees until the process of converting mRNA to cDNA was performed.

Isolation of bacterial

The positive samples for bacterial growth were sub-cultured to get a pure colony, then cultured again on. In order to maintain the broth culture, glycerol was added to a final concentration of 20% and kept at -20°C for 12 to 18 months after the bacterial isolate was inoculated into BHIB broth and incubated for 24 hours at 37°C.

Identification of *Escherichia coli* bacteria

Morphology of the bacteria colony

Bacterial Colony morphology: the expanding colonies on MacConkey Agar Blood base agar and Nutrient Agar was more easily recognized by looking at its morphological features, Gram stain, and appearance under a microscope [20,21].

Using 16S rRNA for bacterial identification and genomic DNA extraction

DNA has been extracted according to (Favorgen Kit). As described in the user's manual, PCR was used to amplify universal bacterial DNA in order to identify the genome of the *E. coli* isolates. 16S rRNA primers for bacteria *E. coli* 27F 5'- AGAGTTTGATCCTGGCTCAG-3' and 1492 R 5'- GGTTACCTTGTTACGACTT-3' [22]. Total volume of 25 µl were used for PCR amplification, F Primer 1 µl, R primer 1 µl, DNA template 3 µl, master mix 12.5µl, Band docter 2.5µl and Nuclease-free water 5 µl were added in the Master Mix tube 25 µl. where the initial denaturation temperature was 95˚C for 5 min with 1 cycle, 30 cycle of denaturation 95˚C for 30 Sec. Annealing 55˚C for 30 Sec, extension 72˚C for 1 min. The final extension was carried out at 72˚C for 5 min with 1 cycle. Three µl of PCR product was loaded inside the well and usually 3 µl of 1000 bp DNA ladder was loaded inside the first well for comparing the size of the amplified gene. The running was for 1 h at 70 volt then the bands were detected under UV transilluminator and photographed.

Sequencing of the 16S rRNA

Sample preparation: the 16S rRNA gene's unpurified PCR products will be sent for purification and analysis. Every sample tube was labeled with numbers that corresponded to excel files that 20µl of PCR product for 16S rRNA was sent to Macrogen in koria.

Gene Sequencing Data Analysis: after being cleaned up and adjusted, the sequence products are copied and pasted into the "BLAST" box, which has been loaded from the http://blast.ncbi.nlm.nih.gov website. 46; E. Coli bacteria names will show up right away along with sequence percentages compared to gene bank data.

Gene expression of ClbA, ClbB and ClbQ gene

Total bacterial RNA extraction

RNA extraction was begin by weighing the quantity (250g) of stool from the patient and the stool was placed in a bead beating tube following an initial dilution with 500 microliters of phosphate-buffered saline (PBS) per sample, the stool-containing tubes were transferred to a chilled environment and vigorously vortexed for 10 minutes each to achieve efficient cell lysis. The extraction steps were then followed using the kit manufactured by Promega Company Every extraction stage was carried out at -4°C. The Nanodrop spectrophotometer was used to measure the amount and quality of RNA.

cDNA synthesis by Reverse Transcriptase Polymerase Chain Reaction (RT-PCR)

Total RNA (300 ng) from each samples was transformed into a cDNA using (TransScript® One-Step gDNA Removal and cDNA Synthesis SuperMix). Since total 4 µl RNA and 3µl nuclease free water, 1µl random primer

(0.1 µg/ µl), 10µl from 2×ES reaction mix, 1µl Easy-Script®RT/RI enzyme mix and 1 µl gDNA remover where mix added to the cDNA master mix tubes that is mixed well by mini vortex and placed in thermal cycler according to program of cDNA synthesis by PCR, primer annealing 25˚C for10 min with cDNA synthesis 42˚C for 15 min and Inactivation enzymes 85˚C for 5 sec.

Real time-PCR

Real time-PCR was performed to estimate the expression of toxins genes using the primers for ClbA forward: AAGCCGTATCCTGCTCAAAA and reverse: GCTTCTTTGAGCGTCCACAT, ClbB forward: GATTTGGATACTGGCGATAACCG andreverse: CCATTTCCCGTTTGAGCACAC,ClbQforward:GCACGATCGGACAGGTTAAT reverse: TAGTCTCGGAGGGATCATGG [23]. SYPR green dye was used to amplify every gene with use reagents used in qPCR. A total volume of 20 µl, 10 µl 2× real-time PCR smart mix, 1 µl forward primer, 1 µl reverse primer, 5 µl templet cDNA, 3 µl nuclease free water with RT-PCR program including, initial denaturation was 95 °C for 15 min with 1 cycle, 40 cycle of denaturation 95°C for 20 sec, annealing 60°C for 40 sec, extension 72°C for 30 sec.

RESULTS

Isolation of bacteria

The 28 bacterial of *E. coli* isolates were extracted from 80 (35%) samples of different sources.

16S rRNA gene amplification of bacterial isolates

Using agarose gel electrophoresis, the 16S rRNA of 28 bacterial isolates was detected; when compared to a molecular DNA ladder, its location was roughly 1500 bp. (Figure 1 and Table 1).

Gene expression of ClbA, ClbB and ClbQ gene

The recent study's findings demonstrated the identification and detection of the most important genes encoding toxins, where the real-time PCR technique by using specific primers for gene expression and SYBR Green dye were used, where the results showed a difference in the expression levels of these genes in most samples through the values shown by Ct. Samples taken from the group of patients with CRC showed high

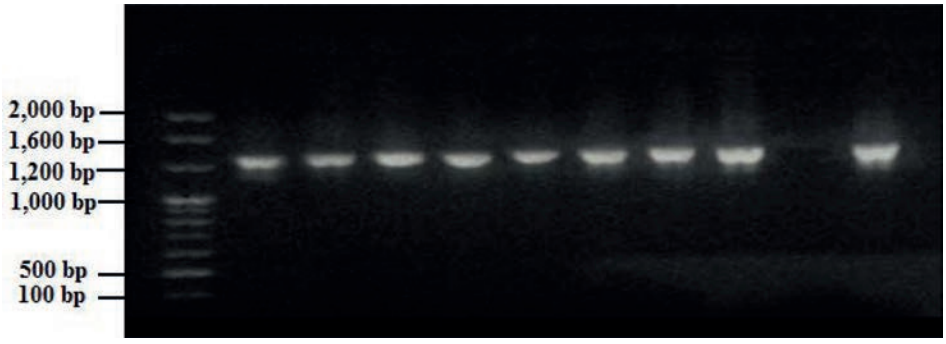

FIGURE 1. *Agarose gel electrophoresis (1.5%) showed a model of amplified 16S rRNA gene (1500bp). Lane L: 100 bp Marker, Lane 1-19: 16S rRNA gene bands for bacterial isolates*

TABLE 1. *Bacteria Escheruchia coli, 16 S rRNA gene nucleotides sequencing, length sources and identity with GenBank strains*

No. isolate	Bacteria species	Nucleotide sequence	Source	Identical to strain	Accession number
28	*E. coli*	TGCAGTCGAACGGTAACAGCGCAACAGTTGCTGCTTCGCT-GACGAGTGGCGGACGGGTGAGTAATGTCTGG-GAAACTGCCTGATGGAGGGGGATAACTACTGGAAACGG-TACTAATACCGCATAACGTCGCAAGACCAAAGAGGGG-GACCTTCGGGCCTCTTGCCATCGGATGTGCCCAGATGG-GATTAGCTAGTAGGTGGGGTAACGGCTCACCTAGG-CGACGATCCCTAGCTGGTCTGAGAGGATGACCAGCCACACT-GGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAG-CAGTGGGGAATATTGCACAATGGGCGCAAGCCTGATGCAGC-CATGCCGCGTGTATGAAGAAGGCCTTCGGGTTGTAAAG-TACTTTCAGCGGGGAGGAAGGGAGTAAAGTTAATACCT-TTGCTCATTGACGTTACCCGCAGAAGAAGCAC-CGGCTAACTCCGTGCCAGCAGCCGCGGTAATACGGAGG-GTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCACG-CAGGCGGTTTGTTAAGTCAGATGTGAAATCCCCGGGCT-CAACCTGGGAACTGCATCTGATACTGGCAAGCTT-GAGTCTCGTAGAGGGGGGTAGAATTCCAGGTGTAGCGGT-GAAATGCGTAGAGATCTGGAGGAATACCGGTGGCGAAGG-CGGCCCCCTGGACGAAGACTGACGCTAGGTGCG	Stool & Blood	%99	LC834134

expression levels for genes, while there was a variation in the control sample group's gene expression., where half of the samples were expressed at lower levels than the healthy group, and the second half of the control group samples were not detected (Table 2).

TABLE 2. *CT values for the genes selected in the study and for the infected sample group and the control sample group*

Gene	Ct value of infection group CRC	Ct value of control groups 1	Ct value of control groups 2	Ct value of Eubacteria
ClbA	25.784	32.874	ND	18.614
ClbB	21.407	28.390	ND	18.614
ClbQ	24.011	32.222	ND	18.614

Estimating of expression level for ClbA gene

Quantitative real-time PCR (qPCR) was used to measure the expression of the ClbA gene, which main part gene that codes colibactin, in study samples. The presence of the ClbA gene was detected in 61% of colorectal cancer (CRC) samples, while it was found in 50% of the first control group and absent in 50% of the second control group. Additionally, the ClbA gene was expressed at a higher level in CRC samples compared to the control groups, as indicated by lower Ct values (Table 2).

Estimating of expression level for ClbB gene

qPCR analysis revealed a high prevalence of the ClbB gene in colorectal cancer (CRC) samples compared to control groups. The gene was detected in 53% of CRC samples, while the first control group showed a 50% prevalence. The second control group exhibited no ClbB gene expression. Quantitative analysis of Ct values indicated significantly higher ClbB mRNA levels in CRC samples, suggesting a potential association with the disease (Table 2).

Estimating of expression level for ClbQ gene

qPCR analysis revealed a high prevalence of the ClbQ gene in colorectal cancer (CRC) samples compared to control groups. The gene was detected in 53% of CRC samples, while the first control group showed a 83% prevalence. The second control group exhibited no ClbQ gene expression. Quantitative analysis of Ct values indicated significantly higher ClbQ mRNA levels in CRC samples, suggesting a potential association with the disease (Table 2).

DISCUSSION

Escherichia coli, the most isolated bacterial species in the study, belongs to the Enterobacteriaceae family,

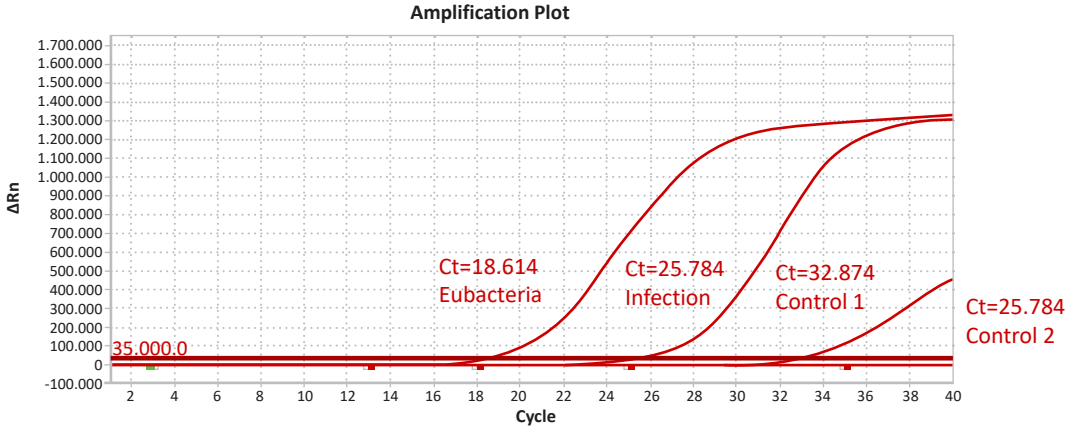

FIGURE 2. *Amplification curve of ClbA gene*

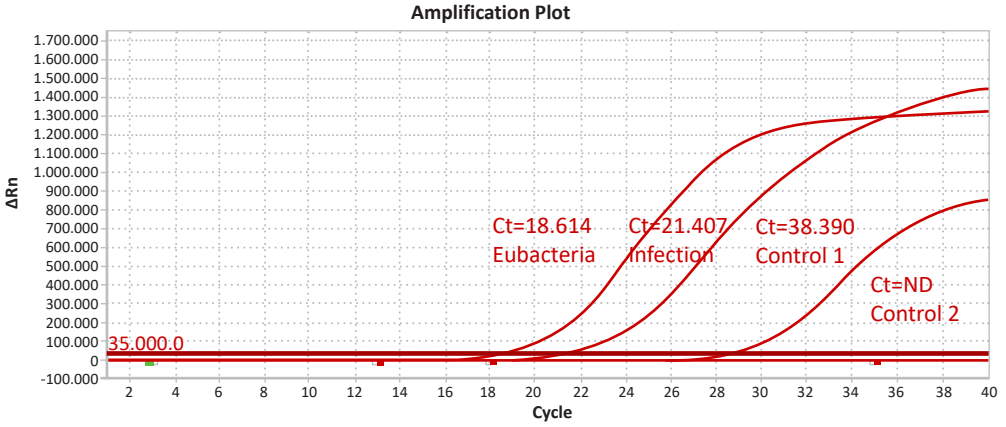

FIGURE 3. *Amplification curve of ClbB gene*

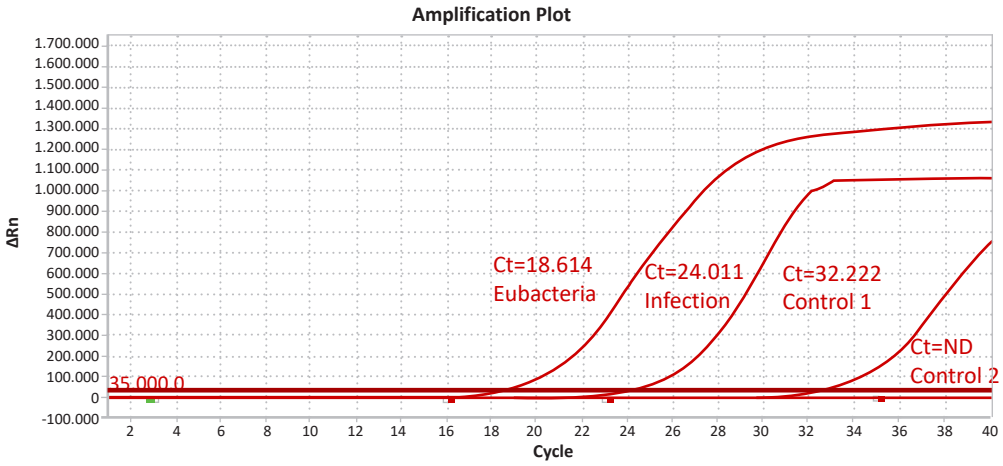

FIGURE 4. *Amplification curve of ClbQ gene*

infects the intestine and causes changes in it that can eventually lead to cellular transformation. Thus, chronic inflammation caused by *E. coli* during inflammatory bowel disease makes the individual more susceptible to colorectal cancer. *E. coli* produces several toxins such as colibactin, which is a hybrid non-ribosomal-polyketide peptide encoded by polyketide synthase (PKS) that can induce the breakage of two strands of DNA, leading to chromosomal aberrations, increasing the frequency of genetic mutations, and capable of stimulating tumorigenesis and contributing to the development of colon cancer [24].

Molecular identification of ClbA, ClbB and ClbQ genes

The present study used RNA sequencing to determine the presence of Escherichia coli in human patient samples (Table 1). The results revealed a high prevalence of *E. coli* in infected individuals, accompanied by high expression of the CblA, ClbB and ClbQ virulence gene encoding colibactin. Real-time PCR analysis confirmed these results (Figures 2, 3 and 4).

To assess the association between expression of CblA, ClbB and ClbQ genes and disease progression, patients were classified into three groups based on CT values (Table 2) infected individuals with high expression of CblA, ClbB and ClbQ genes showed high expression of virulence genes, indicating a higher bacterial load and a possible association with disease severity. Healthy individuals with low expression of ClbA, ClbB and ClbQ genes showed lower levels of expression of CblA, ClbB and ClbQ genes compared to infected individuals, indicating a less significant bacterial presence. The healthy group of individuals with undetectable expression of CblA, clbB and ClbQ genes. This group did not show any detectable expression of the genes, indicating no E.coli colonization or a very low bacterial burden. As the present study showed, most *E. coli* bacteria in all samples contained a polyketide synthase PKS island, and the results were confirmed by the gene expression of ClbB, the main contributor to the expression of colobectin, in addition to confirming our results by the presence of ClbA located near the 5' end and ClbQ located at the 3' end. So, pks island can play a role as carcinogenic risk factor and could be used as a predictive biomarker for cancer development. Because it causes DNA damage, cell-cycle arrest, mutations, and chromosomal instability, the so-called colibactin, a product of the genomic island pks+, might be thought of as a predisposing factor for colorectal cancer. This is further confirmed by its action in *E. coli* cells [25]. Colibactin is a bacterial toxin encoded by pks pathogenicity island which is created by the contribution of 19 genes, which are from the ClbA - ClbS genes, where once the essential genes for colibactin synthesis are present, Usually, environmental stimuli or particular transcription factors inside the bacterial cell control their expression. Regulations governing the production of colibactin are triggered when they detect suitable stimuli, such as metabolites produced by the host or modifications in the microenvironment. This activation results in the strain's pks+ phenotype and the synthesis of colibactin [26], it is one of the main virulence factors of extra-intestinal pathogenic *E. coli* and which can act as genotoxic agent and/or can modulatecellular differentiation, apoptosis, and proliferation [27,28].

Colibactin which is synthetized by polyketide synthases, non-ribosomal peptide synthases, and hybrid enzymes encoded by a 54-kb genomic island pks [29]. It act DNA double-strand breaking, chromosome aberrations, and cell cycle arrest in the G2/M phase [30,31]. It induces instability of chromosome and Broken DNA in cells that are eukaryotic can lead to immunological and epithelial cell senescence apoptosis [29]. Because colibactin alkylates DNA, it can cause interstrand crosslinks and double-strand breaks, which can lead to genetic instability and mutations in important tumor suppressor or oncogene genes. The development of CRC is facilitated by the accumulation of DNA damage, which also helps normal colonic epithelial cells to develop into ma-

lignant tumor cells [30,31].

In addition to starting carcinogenesis, colibactin-induced DNA damage increases the frequency of mutagenesis events and disrupts DNA repair systems, which leads to genomic instability [30]. Where, the organoids' whole-genome sequencing (WGS) revealed that exposure to colibactin produced two distinct mutational signatures: an ID (ID18) with single A or T deletions at poly (dA:dT) tracts known as ID-pks, and a unique single-base substitution (SBS88) signature with T>A and T>C mutations, specifically at ATA, ATT, and TTT motifs [32].

CONCLUSION

These results provide essential basic data that will contribute to understanding the facts of an important part of the tumor formation chain by identifying the pathogenic genes and the mechanisms involved in causing molecular changes at the level of the infected cell. The results revealed a frequency of *E. coli* in infected individuals, accompanied by an increased expression of the virulence genes CblA, ClbB and ClbQ that encode colibactin. Real-time PCR analysis confirmed these results.

Ethical approval

The necessary ethical approval was obtained by verbal consent from patients. The committee gave their approval to this study of publication ethics at Training and Development Center/Basra Health Department from number 16/2021.

Conflict of interest: The authors have disclosed no conflicts of interest.

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Author's contributions:
Conceptualization, A.S.K. and Z.R.A-H.; methodology A.S.K.; software, Z.R.A-H.; validation, A.D.A-H.; formal analysis, Z.R.A-H.; investigation, A.D.A-H.; resources, A.S.K.; data curation, A.D.A-H.; writing—original draft preparation, A.D.A-H.; writing—review and editing, A.S.K.; visualization A.S.K.; supervision, Z.R.A-H.; Project administration, A.S.K.; funding acquisition, A.D.A-H.
All authors have read and agreed to the published version of the manuscript.

REFERENCES

1. Sung H, Ferlay J, Siegel RL, et al. Global Cancer Statistics 2020: GLOBOCAN Estimates of Incidence and Mortality Worldwide for 36 Cancers in 185 Countries. *CA Cancer J Clin.* 2021;71(3):209-49. https://doi.org/10.3322/caac.21660
2. Mahmoud NN. Colorectal Cancer. *Surg Oncol Clin N Am.* 2022;31(2):127-141. https://doi.org/10.1016/j.soc.2021.12.001
3. Zeinab Kamil Dhahi, Ahmed L. Insight on colonoscopy findings in Southern Iraq: Retrospective study from a tertiary center. Iraqi National Journal of Medicine. 2024;6(2):86-90. https://doi.org/10.37319/iqnjm.6.2.5
4. Zhang YJ, Li S, Gan RY, Zhou T, Xu DP, Li HB. Impacts of Gut Bacteria on Human Health and Diseases. *Int J Mol Sci.* 2015;16(12):7493-519. https://doi.org/10.3390/ijms16047493
5. Serino M. Molecular Paths Linking Metabolic Diseases, Gut Microbiota Dysbiosis and Enterobacteria Infections. *J Mol Biol.* 2018;430(5):581-90. https://doi.org/10.1016/j.jmb.2018.01.010
6. Abdul-Hussein ZR. Antibacterial effect of Non-Steroidal Anti-Inflammatory Drugs (NSAIDs). *Bas J Sci.* 2014;32(2B micro conf). https://www.iasj.net/iasj/article/98815
7. Ines M, Annick Bernalier-Donadille, Jubelin G. Enterobacteriaceae in the Human Gut: Dynamics and Ecological Roles in Health and Disease. *Biology.* 2024;13(3):142. https://doi.org/10.3390/biology13030142
8. Cabrera-Sosa L, Ochoa TJ. Escherichia coli Diarrhea. *Hunter's Tropical Medicine and Emerging Infectious Diseases*. 2020;46:481-5. https://doi.org/10.1016/b978-0-323-55512-8.00046-6
9. Poor AP, Moreno LZ, Monteiro MS, et al. Characterization of Escherichia coli Isolated from Sows Presenting Purulent Vulvar Discharge. *Microorganisms.* 2024;12(1):123-35. https://doi.org/10.3390/microorganisms12010123
10. Wang Y, Fu K. Genotoxins: The Mechanistic Links between Escherichia coli and Colorectal Cancer. *Cancers.* 2023;15(4):1152-67. https://doi.org/10.3390/cancers15041152
11. Tariq H, Noreen Z, Ahmad A, et al. Colibactin possessing E. coli isolates in association with colorectal cancer and their genetic diversity among Pakistani population. Woźniakowski G, ed. *PLOS One.* 2022;17(11):e0262662. https://doi.org/10.1371/journal.pone.0262662
12. Akinduti PA, Izevbigie OO, Akinduti OA, Enwose EO, Amoo E. Fecal carriage of colibactin encoding Escherichia coli associated with colorectal cancer among a student populace. *Open Forum Infect Dis.* 2024;11(4):106-17. https://doi.org/10.1093/ofid/ofae106
13. Cougnoux A, Dalmasso G, Martinez R, et al. Bacterial genotoxin colibactin promotes colon tumour growth by inducing a senescence-associated secretory phenotype. *Gut.* 2014;63(12):1932-42. https://doi.org/10.1136/gutjnl-2013-305257
14. Healy AR, Nikolayevskiy H, Patel JR, Crawford JM, Herzon SB. A Mechanistic Model for Colibactin-Induced Genotoxicity. *J Am Chem Soc.* 2016;138(48):15563-70. https://doi.org/10.1021/jacs.6b10354
15. Bossuet-Greif N, Vignard J, Taieb F, et al. The Colibactin Genotoxin Generates DNA Interstrand Cross-Links in Infected Cells. Galan JE, Sansonetti PJ, eds. mBio. 2018;9(2). https://doi.org/10.1128/mbio.02393-17
16. Xue M, Kim CS, Healy AR, et al. Structure elucidation of colibactin and its DNA cross-links. *Science.* 2019;365(6457):2685-2699. https://doi.org/10.1126/science.aax2685
17. Gagnière J, Raisch J, Veziant J, et al. Gut microbiota imbalance and colorectal cancer. *World J Gastroenterol.* 2016;22(2):501-518. https://doi.org/10.3748/wjg.v22.i2.501
18. Post J. Molecular mechanisms through which pathogenic E. coli can contribute to colorectal cancer development. - Student Theses Faculty of Science and Engineering. Ubrugnl. 2023;2023.https://fse.studenttheses.ub.rug.nl/30372/1/Bachelor%27s%20thesis%20Jesper%20Post%20s4111419%20definitive%20version.pdf
19. Holtedahl K, Borgquist L, Donker GA, et al. Symptoms and signs of colorectal cancer, with differences between proximal and distal

colon cancer: a prospective cohort study of diagnostic accuracy in primary care. *BMC Fam Pract.* 2021;22(1):148-161. https://doi.org/10.1186/s12875-021-01452-6
20. Farhan ZA, Al-iedani AA. The phylogenetic groupings of Escherichia coli isolated from human and farm animal feces in Basrah district, Iraq. *NVEO - Nat Volatil Essent Oils J.* 2024;8(5):8982-90. https://www.nveo.org/index.php/journal/article/view/2328
21. Thari AM, Mohammed, Najwa M. J. Abu-Mejdad. Antimicrobial susceptibility of bacterial clinical specimens isolated from Al-Sader Teaching Hospital in Basra-Iraq. *Asia-Pacific J Mol Biol Biotech.* 2024;32(1):76-84. https://doi.org/10.35118/apjmbb.2024.032.1.08
22. Miyoshi T, Iwatsuki T, Naganuma T. Phylogenetic Characterization of 16S rRNA Gene Clones from Deep-Groundwater Microorganisms That Pass through 0.2-Micrometer-Pore-Size Filters. *Appl Environ Microbiol.* 2005;71(2):1084-1088. https://doi.org/10.1128/aem.71.2.1084-1088.2005
23. Piccinini F, Tesei A, Arienti C, Bevilacqua A. Cell Counting and Viability Assessment of 2D and 3D Cell Cultures: Expected Reliability of the Trypan Blue Assay. *BiolProcedures Online.* 2017;19(1). https://doi.org/10.1186/s12575-017-0056-3
24. Shoroug A Al-Harbi, Awatif A. Al-Judaibi. Relationship between Escherichia coli and colon cancer. *GSC Biological and Pharmaceutical Sciences*. 2020;12(2):188-193. https://doi.org/10.30574/gscbps.2020.12.2.0101
25. Piciocchi A, Germinario EAP, Garcia Etxebarria K, et al. Association of Polygenic Risk Score and Bacterial Toxins at Screening Colonoscopy with Colorectal Cancer Progression: A Multicenter Case-Control Study. *Toxins.* 2021;13(8):569-583. https://doi.org/10.3390/toxins13080569
26. Brotherton CA, Balskus EP. A prodrug resistance mechanism is involved in colibactin biosynthesis and cytotoxicity. *J Am Chem Soc.* 2013;135(9):3359-62. https://doi.org/10.1021/ja312154m
27. Arthur JC, Perez-Chanona E, Mühlbauer M, et al. Intestinal Inflammation Targets Cancer-Inducing Activity of the Microbiota. *Science.* 2012;338(6103):120-3. https://doi.org/10.1126/science.1224820
28. Auvray F, Perrat A, Arimizu Y, et al. Insights into the acquisition of the pks island and production of colibactin in the Escherichia coli population. *Microbial Genomics.* 2021;7(5):1-16. https://doi.org/10.1099/mgen.0.000579
29. Faïs T, Delmas J, Barnich N, Bonnet R, Dalmasso G. Colibactin: More Than a New Bacterial Toxin. *Toxins.* 2018;10(4):151-167. https://doi.org/10.3390/toxins10040151
30. Cuevas-Ramos G, Petit CR, Marcq I, Boury M, Oswald E, Nougayrede JP . Escherichia coli induces DNA damage in vivo and triggers genomic instability in mammalian cells. *Proc Natl Acad Sci U S A.* 2010;107(25):11537-42. https://doi.org/10.1073/pnas.1001261107
31. Yu L, Zhao G, Wang L, et al. A systematic review of microbial markers for risk prediction of colorectal neoplasia. *Br J Cancer.* 2022;126(9):1318-28. https://doi.org/10.1038/s41416-022-01740-7
32. Pleguezuelos-Manzano C, Puschhof J, Huber AR, et al. Mutational Signature in Colorectal Cancer Caused by Genotoxic Pks + E. Coli. *Nature.* 2020;580(7802):269-273. https://doi.org/10.1038/s41586-020-2080-8