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Determination of Colibactin Island in Novel *Escherichia Fergusonii* Isolated from Colorectal Cancer Case

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ABSTRACT

Colorectal cancer is the second most common cause of cancer-related death. In our current study, 62 bacterial samples were isolated from 55 biopsies from patients undergoing gastrointestinal endoscopy and surgical procedures. The majority of these biopsies were from patients with colorectal cancer, while the smallest number were from patients with gastric cancer. It is worth noting that 11 colorectal and gastric biopsies failed to culture on culture media. The results showed that *E. coli* was the most prevalent among the Gram-negative isolates obtained, accounting for 46 of the total bacterial isolates.

Escherichia fergusonii was obtained from an elderly woman who had colon cancer and underwent surgery to remove the tumor. Using traditional PCR, Escherichia fergusonii was molecularly identified as one of the isolates that had the colibactin-encoding genes (clbA. clbB, clbN, clbQ). Thus, this was considered the first local and global isolate containing these genes. The 16S rRNA gene sequences were used to prove the diagnosis of *E. fregusonii* and compared with those of the National Center for Biotechnology Information (NCBI).

Deposition of the sequences of 16S rRNA at GenBank, under the accession number PV628355.1, and then the strain was given abbreviated name IAA. The phylogenetic tree was conducted to obtain the relationship between the pv628355 isolate and other Iraqi isolates and the result showed a high relationship between IAA PV628355 with IRQBAS114 LC647818.

Keyword: *Escherichia fergusonii*,16SrRNA, Colibactin. Colorectal cancer

INTRODUCTION

In developing nations, colorectal cancer (CRC) is becoming more common every year. According to Sung et al. (2021), colorectal cancer is the second most common cause of cancer-related deaths globally and the third most common neoplasm. In 2030, it was estimated that the number of people will rise in to 1,1 million with colorectal cancer and about 2.2 million recorded as new additional cases of CRC (Arnold *et al*., 2017). Therefore, research into the causes and mechanisms of colorectal cancer is becoming increasingly important (Qu *et al*., 2022). Numerous studies have demonstrated that gut microbiota plays a critical role in the development of colorectal cancer (CRC) (Wong and Yu, 2019; Karpiński et al., 2022; Long et al., 2019). The gut microbiota can cause cancer by activation of tumor-associated signaling pathways or the production of genotoxins, food metabolism, immunological and inflammatory regulation (Ma *et al*., 2022). According to studies, individuals with (CRC) had higher levels of some harmful bacteria, including *Porphyromonas*, *Escherichia*, *Enterococcus*, *Streptococcus*, *Peptostreptococcus*, *Bacteroides*, *Alternaria*, and *Fusobacterium* (Wang et al., 2012; Yachida *et al*., 2019) .

Escherichia fergusonii is a member of the Enterobacteriaceae Genus *Escherichia* and a have negative reaction with the gram stain. The common sources of isolation are Water (environmental samples) (Maheux *et al*., 2014), animals (Glover et al., 2017), and many clinical samples which include urinary tract infections, human wound infections, pancreatic cancer, bacteremia, diarrhea, and other. (Gokhale *et al*., 2014) .

Previously, in both people and animals, *E. fergusonii* was an uncommon opportunistic infection and referred to as a gut bacteria group, but after many years it was recoded as a new species of Enterobacteriaceae) Farmer *et al*.,1985) which was isolated from a clinical patient. *E. fergusonii* was isolated from the feaces, bile, abdominal wound drainage and blood of a sixty-nine-year-old person who had pancreatic cancer and a bile duct abscess in 1993 (Funke *et al*., 1993). However, following laboratory investigation, confirmed to be the same bacterial clone (Savini *et al*.,2009). Since the intestinal tract is known to be connected to the biliary and pancreatic ducts, it is unclear whether *E. fergusonii* moves from the intestinal tract to the bile canal to cause infection or whether the bile specimens collected are contaminated with intestinal contents (Lai *et al*., 2011).

E. fergusonii have the closest relatives to the *E. coli, E. fergusonii* share over 64% DNA–DNA hybridization with *E. coli*, making it challenging to distinguish between them by using 16S rRNA gene sequencing alone (Farmer *et al*.,1985).

The capacity to ferment lactose and sorbitol by E. coli can be used to distinguish it from *E. fergusonii.* However, it is challenging to determine the *E. coli* O157:H7 strain, because they are unable to ferment sorbitol. Furthermore, incorrect identification can also occur when phenotypic identification is done with an automated tool, like the Vitek 2 automated system (Maheux *et al*., 2014).

The optimum technique for precise discrimination would be phylogenetic analysis with housekeeping genes. Numerous *E. fergusonii* isolates have been shown to be resistant to several antibiotics (Lagacé-Wiens *et al*., 2010).

Numerous AMR genes were found to be present in E. fergusonii which containing some genes known for resistance like gene mcr1, extended spectrum beta-lactamases (ESBLs), ceftriaxone (CTX-M), aminoglycosides (aadA1, strA, strB), ampicillin (TEM-1), , trimethoprim (dfrV, dfrA1), beta-lactamase resistant genes (blaCMY2, blaTEM, blaACT, blaSHV, blaCTX-M-15), tetracyclines (tetA, tetB, tetC, tetE), sul1, sul2, , and carbapenemases. According to earlier research, the bacteria may also have a number of virulence markers, such as heat-labile toxins, heat-stable toxins, depending on their virulence genes, such as eae,iss, prfB, and ireA, which were all known to exist in *E. coli* (Bhowmik *et al*., 2025) .

Aim of study

Determining the phylogenetic relation between Colibactin positive *E. fregusonii* strain isolated from colon cancer biopsy with other *E. fregusonii* Iraqi strains isolated from different clinical samples and focusing on this strain which is regarded as the novel strain have colibactin genes.

MATERIALS AND METHODS

Sampling

The study included 55 tissue biopsies collected from 55 patients (30 females and 25 males) who were diagnosed with or suspected to have colorectal cancer and gastric cancer, excluding other types of cancer. The females were aged between 17-77 years, while the males were aged between 24-86 years. They visited the gastrointestinal endoscopy department at Al-Jamhuri, Ibn Sina and Al-Bahthi Educational Hospitals/ Mosul/ Iraq in addition to tissue biopsies from surgical operations for patients with gastric cancer and colorectal cancer from Al-Jamhuri Hospital/ Mosul/ Iraq.

Isolation and identification

A glucose solution (20%) was used as a transport medium for biopsy samples. The samples were transferred into the laboratory under cooling conditions (ice box). Samples were crushed using a ceramic mortar sterilized with alcohol and then cultured in Luria-Bertani broth in duplicate, incubated at 37°C for 18–24 hours. A loopful of LB broth was then taken and inoculated onto MacConkey agar and blood agar, and incubated at 37°C for 24h. Suspect colonies were taken, then cultured several times on MacConkey agar until pure cultures were obtained. Diagnosis was first made using Gram stain. Pure isolates were then preserved at −80°C (using 20% glycerol) (Tariq *et al*.,2022). To confirm the diagnosis, samples were sent to the VITEK II system.

DNA extraction and quantification

A commercial kit (Presto TM Mini gDNA Bacteria/ Geneaid) was used to extract the genomic DNA from our isolate. In short, 180 µL of GT Buffer was added to a 1.5 microcentrifuge tube containing several loopful of bacterial colonies, and the cell pellets were resuspended using a pipette or vortex. After adding 20 µL of proteinase K, the mixture was incubated for at least ten minutes at 60°C. The tube was inverted every three minutes during the incubation period. After adding 200 µL of GB Buffer, the material was vortexed for ten minutes.

To guarantee clarity, the bacterial cell extract was then incubated for ten minutes at 70°C. The tube was inverted every 3 minutes during the duration of incubation. After adding 200 µL of 100% ethanol, the testing solution was given a good shake. A 2-ml collecting tube was used to hold the GD column. After being moved to a GD column, the insoluble precipitate was centrifuged for two minutes at 14,000–16,000 xg. Then 2ml collection tube was used to dispose of the leftovers. Another new 2ml collection tube was filled with the GD column.

The GD column was filled with 400 µL of W1 buffer solution, and it was centrifuged for thirty seconds at 14,000–16,000 x g. After the residue was eliminated, the GD column was put back into a 2 mL collection tube, 600 µL of wash buffer solution was added, and it was centrifuged for thirty seconds at 14,000–16,000 xg. After that, the residue was taken out, put back in a fresh 2 mL collection tube, and the column matrix was dried by centrifuging it for 3 minutes at 14,000–16,000x g.

A fresh 1.5 mL microcentrifuge tube was filled with the dried GD column. In the middle of the column matrix was the heated elution solution. To purify the elution, it was centrifuged for 30 minutes at 14-16,000 x g after 3 minutes (to allow the elution solution to be fully absorbed). After the genomic DNA's concentration and purity were assessed, it was stored at -20 °C in an Eppendorf tube until it was needed.

Polymerase chain reaction (PCR) protocol

A colibactin genomic island, was screened by polymerase chain reaction (PCR) in the clinical samples. Primers for the *clb*A, *clb*B, *clb*N, and *clb*Q genes were used for amplification, as indicated in (Table 1).

Promega/USA supplied the GO Taq G2 Green Master Mix, which was used to conduct PCR experiments. 1 µl of reverse and forward primers, 10 µl of green master mix, template DNA added in accordance with their quantities, and the remaining volume was nucleotide-free water were all added to a 20 µl PCR reaction volume. Polymerase chain reaction conditions were 95 °C for 15 minutes (35

cycles) for *clb*A, B, N, and Q, and 35 cycles for the other stages as follows: 30 sec. at 95°C, 30 sec. at 53°C, and 90 sec. at 72°C. Lastly, a 10 min. cycle at 72 °C (Table 2). (Hussein *et al.,* 2020).

Table (1): Primers for single PCR of colibactin island (Hussein *et al.*, 2020)

Gene	Primer (5'-3')	Target size or PCR product size (bp)
*clb*A	F- CTAGATTATCCGTGGCGATTC	1002
	R-CAGATACACAGATACCATTCA	
*clb*B	F-GATTTGGATACTGGCGATAACCG	550
	R-CCATTTCCCGTTTGAGCACAC	
*clb*N	F-GTTTTGCTCGCCAGATAGTCATTC	700
	R-CAGTTCGGGTATGTGTGGAAGG	
*clb*Q	F-CTTGTATAGTTACACAACTATTTC	821
	R-TTATCCTGTTAGCTTTCGTTC	

Table (2): Polymerase chain reaction conditions used to amplify the genes encoding colibactin.

Steps	N. of cycles	Temperature ºC	Time
Pre-denaturation	1	95	15:00 min
Denaturation	35	95	0:30 sec
Annealing		53	0:30 sec
Extension		72	1:30 min
Final Extension	1	72	10:00 min

Gel electrophoresis

2% agarose was used for running the PCR products. One gram of agarose was dissolved in fifty milliliters of 1x TAE, heated to a boiling point, and then cooled to forty-five degrees Celsius. At this degree add 0.8 µl weight /50 ml volume of Safe Red to agarose solution. Then, the solution was mixed. For comparison, PCR products were loaded in agarose wells at a size of 5 µl , and a 6 µl DNA ladder was added to the first well. 50–100 V for 50 min was one of the electrophoresis parameters used to mobilize the DNA. UV light was used to visualize the PCR products (AL-Obady 2022).

Identification using the 16S rRNA technique

The *16S rRNA* gene is used to identify the bacterial isolates, the partial segment of the *16S rRNA* gene (1495 bp) was amplified from the genomic DNA of the bacterial strain, using the 27F and 1522R universal primers listed in (Table 3). The PCR conditions for amplifying the 16S rRNA gene were conducted as shown in (Table 4). (Khaleel *et al.,* 2023)

Table (3): Primers used in amplification of the 16S rRNA gene

Primer	Primer sequence (5' to 3')	Product size (bp)	Reference
27F 1522R	AGAGTTTGATCTGGCTCAG AAGGAGGTGATCCARCCGCA	1465	(Khaleel *et al*., 2023).

Table (4): Polymerase chain reaction conditions used to amplify genes 16S rRNA

Steps	N. of cycles	Temperature ºC	Time
Pre-denaturation	1	95	3:00 min
Denaturation	30	95	0:30 sec
Annealing		55	0:30 sec
Extension		72	1:00 min
Final Extension	1	72	3:00 min

Evolutionary analysis by maximum likelihood method

The phylogeny was done using the Maximum Likelihood method. The phylogenetic relationship built between our isolate PV628355.1 and the isolates recorded in NCBI data base that isolated from Iraq belong to different researchers from other studies (P808029.1, P808022.1, LC648290.1, LC647818.1, PV628355.1, LC647822.1, LC856521.1, PP526953.1, P808031.1, PP526966.1, PV162887.1, PQ458504.1). The initial tree for the heuristic search was selected by choosing the tree with superior log-likelihood between the Neighbor–joining (NJ) tree and a Maximum Parsimony (MP) tree. The analytical procedure encompassed 12 coding nucleotide sequences using 1st, 3rd, and non-coding positions with 1,446 positions in the final dataset. Evolutionary tree was constructed depending on MEGA12 (Tamura, 1993; Satiou,1987; Nei and Kumar ,2000).

RESULTS AND DISCUSSION

In the current study, we obtained a higher percentage of isolates from colorectal biopsies than from gastric biopsies, as 51 bacterial isolates were obtained from colorectal biopsies, and 11 bacterial isolates from gastric biopsies. The results showed that the prevalence of *Escherichia coli* was the largest among the gram-negative isolates, which amounted 46 isolates (74.19%) among the total bacterial isolates, in addition to one isolate of *Escherichia fergusonii*. Other gram-negative bacterial isolates included 3 isolates (4.83%) of *Klebseilla pneumoniae* and one isolate (1.6%.) obtained from the following species: *Enterobacter hormachei, Providencia alcalifaciens, Sphingomonas puncimobilis, Pseudomonas aeruginosa, Enterobacter cloacae, Morganella morganaii,* and *Stenotrophomonas geniculate*.

The results of our study showed that the number of patients with colorectal and stomach cancer reached (34) at a rate of (61.81%) out of a total of 55 patients who visited the endoscopy unit, 26 of them were patients with colorectal cancer at a rate of (76.47%), and 8 patients with stomach cancer at a rate of (23.52%). Our results also showed that the ages of males with colorectal cancer ranged between (31-86 years), while the ages of females ranged between (35-77) years. The number of females with colorectal cancer was greater than the number of males. As for the age of people with stomach cancer, the ages of males ranged between (40– 74 years), while the ages of females ranged between (34- 64 years). The number of females and males with stomach cancer was equal. It was found that the age group of colorectal and stomach cancer among males and females was (50 -70) years, as in Fig. (1) and Fig. (2). The number of biopsies taken from healthy individuals was 21, with an average of 16 biopsies from healthy individuals with colorectal 9 males aged between (24 - 83) and 7 females aged between (17 – 70). As for stomach biopsies, they were taken from five individuals (4 females aged between 30 -72) and one male aged 70 years. The number of isolates was taken from healthy individuals 22 isolates.

One isolate *Escherichia fergusonii* was obtained from an elderly woman who had colon cancer and underwent surgery to remove the tumor.

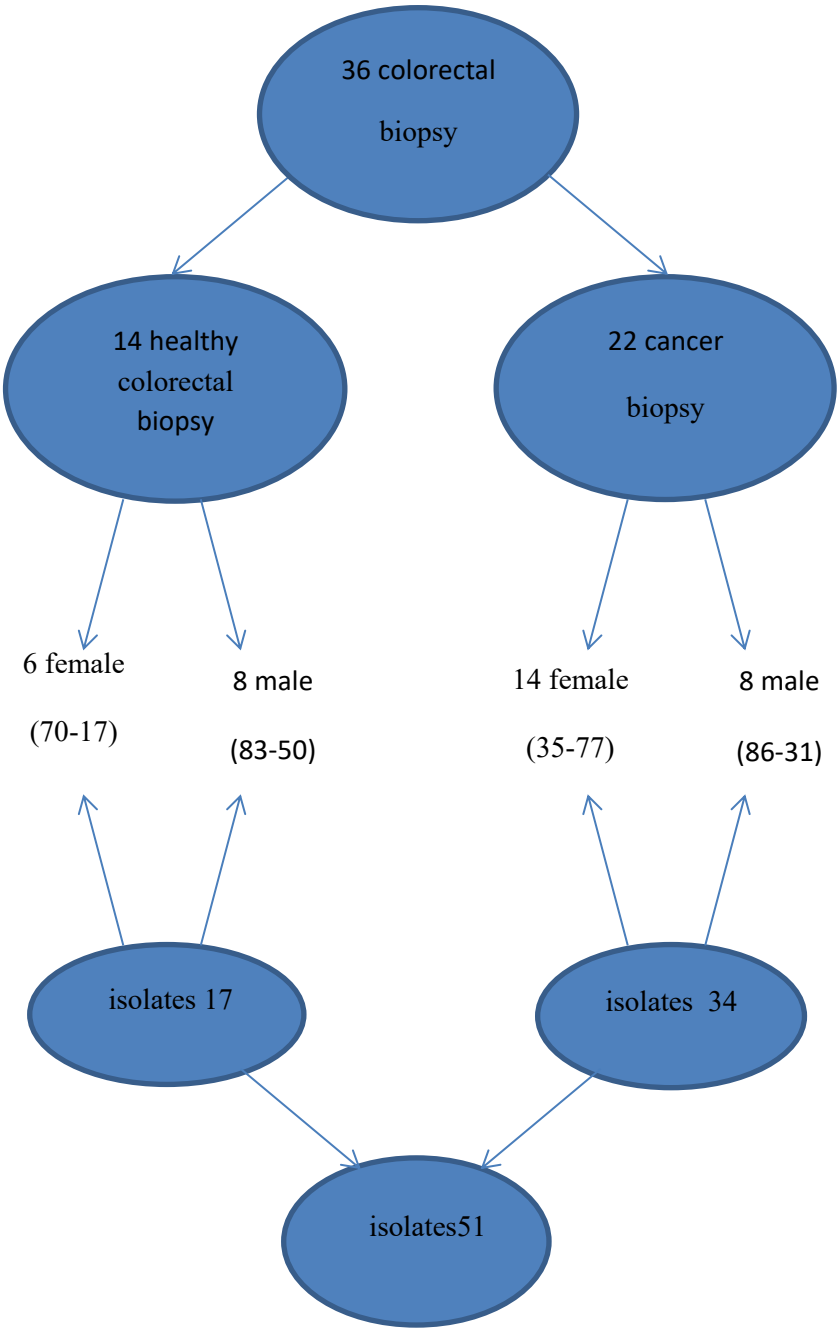


Fig. 1: Distribution of colorectal isolates according to the type of sample, age and gender

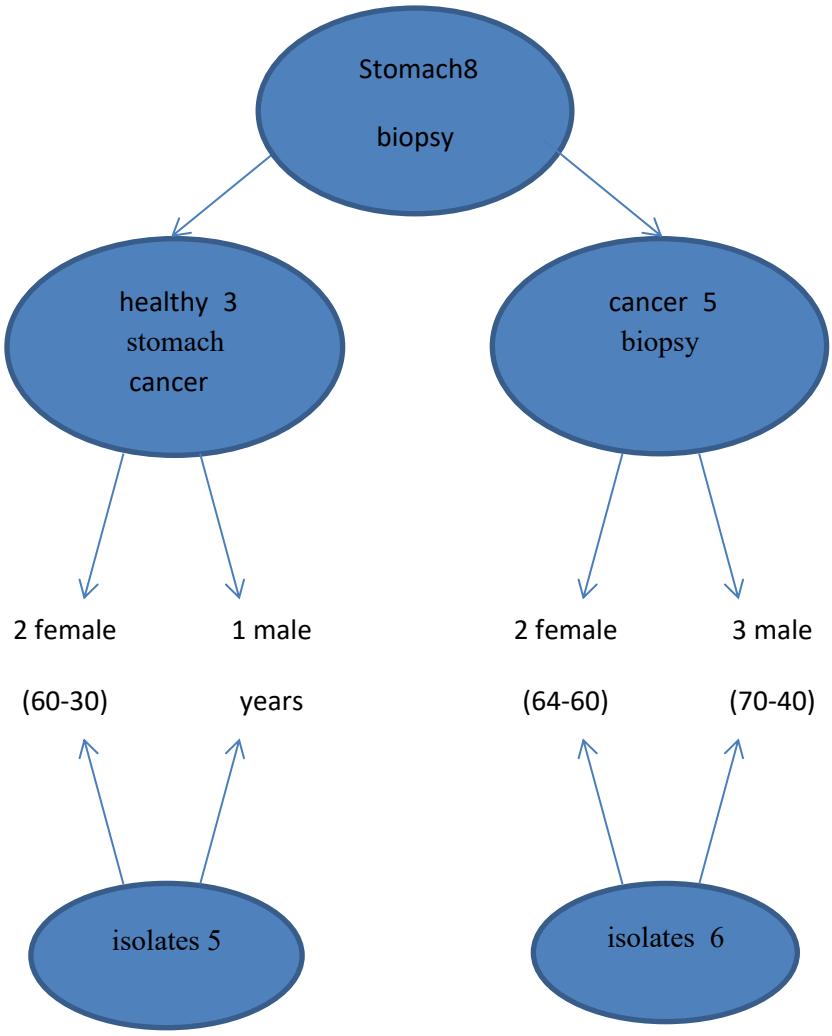
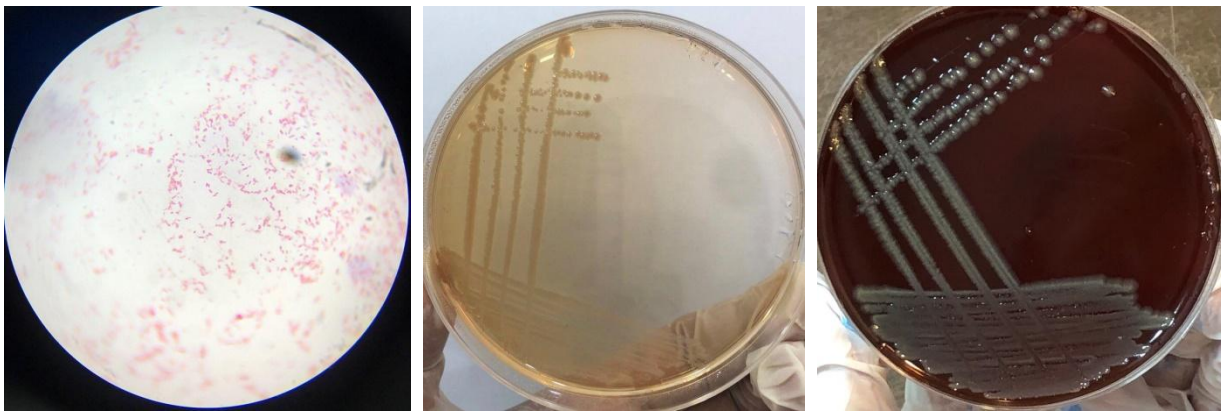


Fig. 2: Distribution of stomach isolates according to sample, age and gender

The initial diagnosis for our isolate was based on the shape of the cells by using light microscopic examination that revealed gram negative, rod shape bacteria with pale colonies on MacConkey agar, and were round, moist, and grayish or off-white in color, non-haemolytic on blood agar Fig. (3: A, B, C).



A B C

Fig. 3: A: *E. fergusonii* is a gram-negative bacterium (100x), B: *E. fergusonii colony* on MacConkey agar, C: *E. fergusonii colony* on Blood agar

The diagnosis by the Vitek II device showed a variety in identification and retained it to *E. coli* species, although absent the ability to ferment lactose by this strain. To confirm identification 16S rRNA was used for good vision, and the result was that it was an *E. fergusonii* strain Fig. (4).

Escherichia fergusonii strain IAA 16S ribosomal RNA gene, partial sequence

GenBank: PV628355.1

FASTA Graphics

Go to:

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VERSION     PV628355.1
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  ORGANISM  Escherichia fergusonii
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            Enterobacterales; Enterobacteriaceae; Escherichia.
REFERENCE   1  (bases 1 to 778)
  AUTHORS   Matter,I.R., Al-rawi,A.M. and Almola,A.H.
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  JOURNAL   Unpublished
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  AUTHORS   Matter,I.R., Al-rawi,A.M. and Almola,A.H.
  TITLE     Direct Submission
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      661 tagagatctg gaggaatacc ggtggcgaag gcggcccccc ggacgaagac tgacgctcag
      721 gtgcgaaagc gtggggagca aacaggatta gataccctgg tagtccacgc cgtaacga
//
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Fig. 4: Confirm Identification 16S rRNA

When using the genomic DNA extraction kit to extract the DNA, the concentration was 128.1 ng/µl and the purity was 1.777. In our study the presence of *clbA, clbB, clbN* and *clbQ* island colibactin genes was detected by using traditional PCR reaction depending on the specific primers for it, and then the size of the product was observed under UV illumination after electrophoresis on 1.5 agarose gel as 1002bp for *clbA* ,550bp for *clbB*,700bp for *clbN*, and 821bp for *clbQ*. Fig. (5).

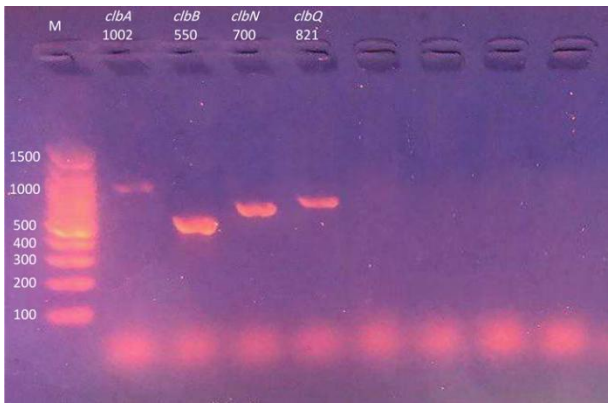

Fig. 5: 2% agarose DNA gel of *clb* genes in *E. fergusonii*, and visualized by UV light.

The 16S rRNA gene sequence was done for *E. fregusonii* and the results of the analysis were recorded in the data of the NCBI for the purpose of comparison. The sequences of 16S rRNA were installed into GenBank, and gave the accession number PV628355.1. The strain had given abbreviated name IAA Fig. (6). The phylogenetic tree showed a high relationship between our isolate with IRQBAS114 LC647818 Fig. (7)

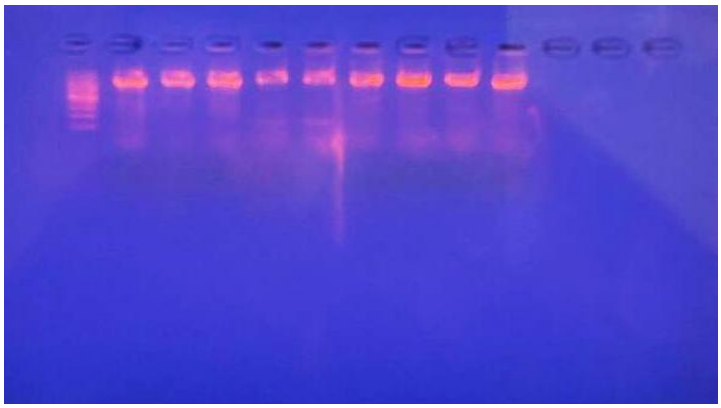

Figure 6. Gel electrophoresis of 16SrRNA product

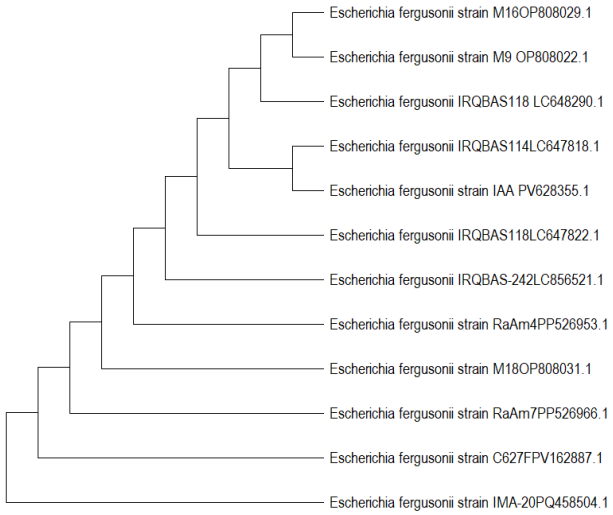

Figure 7. Evolutionary analysis by the Maximum Likelihood method

The isolation results clearly show that *E. coli* was the most common. This could be because of its pathogenic factors and capacity to cause genotoxicity in cells. Cyclomodulin were the bacterial

toxins that cause this genotoxic impact (Nougayrède et al., 2005). By causing DNA damage and genomic instability, cyclomodulins disrupt the eukaryotic cell cycle, which can lead to tumorigenesis. (Gagnière *et al*., 2016)

Colibactin was identified by Nougayrede and colleagues as a genotoxin belonging to cyclomodulins in 2006 (Nougayrède *et al*., 2006). It is a small toxin encoded by a 54-kilobase genomic island found in Enteriobacteriaceae such as *E. coli* which is regarded as a normal flora in the human intestinal tract. Colibactin may be related to cancer of the colon (Nougayrède *et al*., 2005).

One isolate of *E. fergusonii* was obtained from 62 isolates and it proved pks+ island by using genotyping of it, and this is the first globally and locally reported result that prove *E. fergusonii* possesses the genomic pks island (clbA,*clb*B,*clb*N and *clb*Q genes) that is responsible for the production of colibactin toxin which plays a significant role in colon cancer. This result reflects the high rate of gene transfer between gram-negative bacteria especially Enterobacteriaceae.

The product of pks included many essential enzymes for the manufacturing of various compounds such as antibiotics, and many molecules have biological effects and very important in fatty acid synthesis. (Torres *et al*., 2020)

As was already noted, of the isolates that were obtained, *E. coli* had the highest prevalence. This is confirmed by (Hussein *et al*, 2020), who found that *E. coli* had the largest share among the various isolation sources, constituting 58% (51 isolates) of the 88 isolates belonging to the Enterobacteriaceae family. Also, (Martinson and Walk, 2020) confirmed that *E. coli*, a facultative anaerobic Gram-negative bacterium, is present in the intestinal microbiome of more than 90% of humans. Along with specific pathogenic strains, E. coli has a symbiotic relationship with its host and performs countless vital roles in the human gut, such as vitamin K production (Suvarna *et al*., 1998) and protection against intestinal pathogens (Richter *et al*., 2018). E. coli is a common component of the human microbiome (Dougherty and Jobin, 2023).

Enterobacteriaceae, such as *E. coli, Klebsiella pneumonia*, Enterobacter spp and *Morganella morganii* have been associated with CRC (Cao *et al*.,2022; Yurdakul *et al*.,2015; Strakova et al.,2021; Wassenaar 2018). Our study also identified a higher diversity of microorganisms in the CRC patient samples when making a comparison with the control. In addition, Enterobacteriaceae were detected in higher ratio in the samples of the CRC patients compared with healthy people.

Our study also agreed with the study by Bachelle *et al*., (2024) in that gut bacteria isolated from colorectal cancer patients were more diverse compared to isolates taken from healthy participants, thus colorectal cancer is linked to bacterial imbalance. At the same time, our study differs from the same study in that the pks genomic island (clbA-clbS), which has been reported to be overrepresented in the gut bacteria from colorectal cancer patients, in contrast to the result of Bachelle study in which Enterobacteriaceae isolates did not have pks genomic island from the study participants. Analysis of the unique genes of E. coli isolates from colorectal cancer patients, which carried virulence genes in high numbers, it gave us an idea that several mechanisms and virulence factors other than colibactin may be contributing to the development of colorectal cancer. In addition, Enterobacteriaceae carrier genes of cyclomodulin may already be present in the intestines of cancer patients which increase the risk of CRC for these persons. These *E. coli* virulence genes were also identified in the genomes of *E. fergusonii*, which confirms the importance of horizontal gene transfer between bacteria in the intestinal tract.

Our study differs from Putze *et al*., (2009), who found *E. fergusonii* isolate did not contain colibactin genes, while at the same time found 76 isolates from *E. coli* were positive for colibactin genes.

In conclusion, the ability of certain Enterobacteriaceae to produce genotoxins like colibactin, mediated by the pks genomic island, is a key factor in their pathogenicity and their potential role in colorectal carcinogenesis. The high prevalence of pks+ strain in the human gut microbiome and its dual role as both a symbiont and potential pathogen underscores the complex interactions between host and microbiota in health and disease.

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تحديد جزيرة الكوليباكتين في عزلة فريدة من *Echerichia fergusonii* معزولة من سرطان القولون والمستقيم

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الملخص

يُعد سرطان القولون والمستقيم ثاني أكثر أسباب الوفيات المرتبطة بالسرطان شيوعًا. في دراستنا الحالية، جُمعت 62 عينة بكتيرية معزولة من 55 خزعة من مرضى راجعوا قسم تنظير الجهاز الهضمي ومن عمليات جراحية . كان النصيب الاكبر من هذه الخزع من مرضى القولون والمستقيم، والعدد الاقل من الخزع من نصيب مرضى المعدة. ومن الجدير بالذكر ان 11 خزعة من القولون والمستقيم ومن المعدة لم تعطي نتيجة ايجابية عند زراعتها على الاوساط الزرعية. وأظهرت النتائج أن بكتريا *E. coli* كانت الاكثر انتشارا بين العزلات السالبة لصبغة كرام المحصل عليها، اذ بلغت 46 عزلة من اجمالي العزلات البكترية.

تم الحصول على عينة من *Echerichia fergusonii* من امرأة مسنة مصابة بسرطان القولون وخضعت لعملية جراحية لإزالة الورم .تم التعرف على *Echerichia fergusonii* جزيئيًا باستخدام تفاعل البوليميراز المتسلسل التقليدي، من بين العينات التي تحتوي على الجينات المشفرة للكوليباكتين (*clb*A.*clb*B,*clb*N,*clb*Q). وبالتالي، تُعتبر هذه أول عينة محلية وعالمية تحتوي على هذه الجينات .استُخدمت تسلسلات جينات *16SrRNA* لإثبات تشخيص *Echerichia fergusonii*، وقورنت بتسلسلات المركز الوطني لمعلومات التكنولوجيا الحيوية (NCBI). تم تسجيل تسلسلات *16SrRNA* في بنك الجينات، تحت رقم الدخول PV628355.1، ثم أُطلق على السلالة اسم pv628355 المختصر .تم رسم شجرة النشوء والتطور لتحديد العلاقة بين عزلة IAA وعزلات عراقية أخرى، وأظهرت النتيجة علاقة وثيقة بينها وبين IRQBAS114 LC647818

الكلمات المفتاحية: *Escherichia fergusonii*، 16SrRNA، كوليباكتين، سرطان القولون والمستقيم.