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A novel evidence of *Providencia alcalifaciens* possessing the genotoxin colibactin

I. R. Matter, A. M. Alrawi, A. H. Al-Mola

University of Mosul, Mosul, Iraq

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Department of Biology, College of Science, University of Mosul, Mosul, Iraq. E-mail: rayanmazin@uomosul.edu.iq

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Cancer is one of the most threatening diseases to human health in the world. Consequently, this study was dedicated to shed light on an important part of this trend. Fifty five biopsies were collected from various sources, including stomach, colon, and rectal biopsies from the Gastrointestinal Endoscopy Department in Ibn Sina, Al-Jumhuri and Al-Bahth Hospitals, and surgical operations samples from patients with stomach and colon cancer from Al-Jumhuri Hospital and Al-Hayat Private Hospital in Mosul city (Iraq). Among 61 bacterial isolates obtained in our study, they were all diagnosed as *E. coli* except one isolate diagnosed as *Providencia alcalifecians* as based on morphological examination and a Vitek II device. It was found that a high percentage of isolates carried the island genes (*clbA*, *clbB*, *clbN*, and *clbQ* genes) for colibactin toxin (genotoxin), which causes host cell DNA damage and breaks, with 33 isolates possessing all the genes. *P. alcalifaciens* contained all genes studied depending on the PCR result. Our results show for the first time locally and globally the presence of genomic islands in *P. alcalifaciens* responsible for the production of colibactin toxin. The presence of these genes had not previously been diagnosed in such a potential pathogen.

Keywords: *Providencia alcalifaciens*; colibactin toxin; colon cancer; *clb* genes.

Introduction

When Fearon and Vogelstein first described colorectal carcinogenesis as a classic adenoma-carcinoma sequence in 1990, they identified a number of risk factors that favor this tumoral development, and pathogens appear to be a major one among them; in fact, infectious agents are responsible for about 20% of the world's cancer burden. Bacteria and their byproducts may contribute to the development or advancement of sporadic colon cancer through a number of mechanisms, such as the induction of proinflammatory and procarcinogenic pathways in epithelial cells, the generation of reactive oxygen species and genotoxins, and the transformation of procarcinogenic dietary factors into carcinogens. Numerous bacteria, such as *Helicobacter pylori*, *Streptococcus bovis*, *Enterococcus species*, the enterotoxigenic *Bacteroides fragilis*, and several pathogenic *Escherichia coli*, have been linked to the development of colorectal cancer (Bonnet et al., 2014).

Tumors have different microbial communities than the surrounding healthy tissue. Additionally, the tumor microenvironment's microbial diversity expanded, as seen by shifts in the abundances of pathogenic and commensal bacterial taxa, such as *Providencia*. *Providencia* is a novel tumor-associated agent that had not been detected in earlier studies. Furthermore, researchers have discovered a distinct and noteworthy enrichment of anticipated virulence-associated genes in the microenvironment of colorectal cancer, which is probably reliant on the genomes of *Providencia* (Burns et al., 2015).

It is intriguing that *Providencia* was found in the tumor microbiome, it encodes a strong, immunogenic lipopolysaccharide. Indeed, this genus shares a number of virulence genes that are involved in the manufacture of lipopolysaccharides, and it is similarly markedly elevated in the tumor microenvironment. Furthermore, it has been demonstrated that *Providencia* damages the intestinal epithelial membrane, albeit it is yet unknown how this is achieved (Burns et al., 2015).

Providencia species are among the least investigated members of the Enterobacteriaceae family, while being part of a sizable and clinically significant family of Gram-negative bacteria. *Providencia* species inhabit a variety of habitats and hosts. they are present in the human gut, oral cavity, and sputum microbiomes (Baker et al., 2019; Zambruni et al., 2019), as well as in soil (Ferraresso et al., 2020), water (Gessew et al., 2022), sewage, and retail meats, fruits, and vegetables (Klein et al., 2024). *Providencia* rod-shaped bacteria currently has 9 species, the most common species are *P. stuartii*, *P. rettgeri*, *P. rustigianii*, and *P. alcalifaciens*, which are responsible for a variety of infections, including septicemia, nosocomial infections, diarrhea, wound infections, and urinary tract infections (Shah et al., 2019). *P. alcalifaciens* is a bacterium that is regarded as negative for lactose that forms pale colonies on enteric agars including *Salmonella-Shigella* agar, MacConkey agar, and deoxycholate citrate agar.

API-20E commercial biochemical strip kit (bioMerieux, Marcy-L'Etoile, France), and automated systems, such as Vitek-II (bioMerieux), can be used to identify *P. alcalifaciens* based on its biochemical responses. Despite being regarded as a normal component of the fecal flora, *P. alcalifaciens* has been shown to induce diarrhea in a number of species, including humans. It has been linked to traveler's diarrhea and foodborne diarrhea epidemics (Bulach et al., 2024).

The research aims to provide new evidence locally and globally about the presence of *P. alcalifeciens* isolated from gastrointestinal endoscopy biopsies of patients with colon cancer, which possesses the genomic island responsible for the production of colibactin, using molecular methods.

Materials and methods

55 biopsies were collected from different sources, including stomach, colon and rectal biopsies from the Gastrointestinal Endoscopy Department at Ibn Sina, Al-Jumhuri and Al-Bahth Hospitals, in addition to samples from surgical operations for patients with stomach, colon and rectal cancer from Al-Jumhuri Hospital and Al-Hayat Private Hospital in Mosul city (Iraq).

Glucose solution (20%) was used to preserve biopsy samples for a short time and for transporting it to laboratory by ice box. The samples were homogenized and cultured on Lauria Bertani broth in duplicates, and incubated for 18–24 hours at 37 ˚C, then a loopful was taken from the LB broth and inoculated on MacConkey agar and blood agar, incubated at 37 ˚C overnight. The suspected colonies were taken, subcultured on MacConkey agar. The diagnosis was done using a single colony for conducting Gram's staining. The preservation of isolates were achieved by storage at –80 ˚C in 20% glycerol (Tariq et al., 2022). Diagnosis of bacterial isolates were confirmed via VITEK-2 system.

Genomic DNA of our isolates was obtained using a commercial kit (PrestoTM Mini gDNA Bacteria/Geneaid). Briefly, we transferred many samples of bacteria colonies to a 1.5 microcentrifuge tube which contained 180 µL of GT Buffer then re-suspended the cell pellet by vortex or pipette, added 20 µL of Protinase K, incubated at 60 ˚C for at least 10 minutes. During incubation we inverted the tube every 3 minutes, added 200 µL of GB Buffer to the sample and mixed by vortix for 10 minutes, incubated at 70 ˚C for 10 minutes to ensure the sample lysate was. clear.

Absolute ethanol was added ast200 µL to the lysate of sample and mixed by shaking vigorously. The GD column put in a 2 mL collection tube. Insoluble precipitate was transferred to the GD column and centrifuged for 2 minutes at 14,000–16,000 g. The residuals in the 2 mL collection tube were discarded. The GD Column was placed in a new 2 mL collection tube.

400 µL of W1 Buffer was added to the GD column and centrifuge for 30 seconds at 14000–16000 g. The residual contents were discarded and the GD column replaced into the 2 mL collection tube and 600 µL of wash buffer was added to the GD column and centrifuged for 30 seconds at 14000–16000 g. The residual contents again were discarded and it was placed back in the new 2 mL collection tube and centrifuged for 3 minutes at 14000–16000 g for column matrix drying. The dried GD column was transfered to a new 1.5 mL microcentrifuge tube. Pre-heated elution buffer put into the center of the column matrix. After 3 minutes (to allow the elution buffer to be completely absorbed) centrifuging was done for 30 minutes at 14000–16000 g for purified DNA elution. Genomic DNA purity and concentration were measured, and after that, DNA was placed in an Eppendorf tube at –20 °C until its use.

The presence of colibactin genomic islands known as polyketide synthase (pks) islands in the collected clinical isolate was determined by Polymerase Chain Reaction (PCR). The amplification was performed using primers for *clbA*, *B*, *N* and *Q* genes listed in Table 1. PCR was done using GoTaq G2 Green Master Mix (Promega, USA). Reaction volume of PCR was performed in 20 µL containing 10 µL of master mix, 1 µL of reverse and forward primers, 1 µL template DNA, and 7 µL nuclease free water. PCR conditions for *clbA*, *B*, *N*, and *Q* were 95 ºC for 15 min (35 cycles) and 35 cycles for other steps as following: 30 s at 95 ºC, 30 s at 53 ºC, and 90 s at 72 ºC. Finally, 1 cycle of 10 min at 72 ºC (Hussein et al., 2020).

The products of PCR were run in 2% agarose. Agarose solution was prepared by dissolving 1 gram of agarose in 50 mL of TAE 1X, then heated until boiling followed by cooling it at 45 °C. The red safe pigment was added to the solution at 0.8 µL / 50 mL volume. DNA ladder was added as 6µl into the first well for comparison purposes with PCR products loaded into other wells as 5 µL. The conditions for electrophoresis included 50–100 volts for 50 minutes for DNA movement. The products of PCR were illuminated under UV for visualization (Khaleel et al., 2023).

Results

Among 55 biopsies collected, 61 bacterial isolates were obtained from healthy and suspected cancer patients, most of these isolates were *Escherichia coli.* There was only one isolate belonging to the genus *Providencia,* which was diagnosed as *P. alcalifaciens* from an elderly woman with colon cancer. The initial diagnosis for our isolate was based on the morphology of the cells by microscopic examination that revealed very small rods with a pleomorphic or coccobacilli shape (Fig. 1) and showed small pale colonies when grown on MacConkey agar, small transparent colonies on nutrient agar, and were round, moist, and grayish or off-white in color, non-haemolytic on blood agar (Fig. 2). The diagnosis was confirmed using the Vitek II system.

In our study the presence of *clbA*, *clbB*, *clbN* and *clbQ* island colibactin genes were detected using traditional PCR reaction depending on specific primers. The size of the products was observed under UV illumination after electrophoresis on 1.5% agarose gel as 1002 bp for *clbA*, 550 bp for *clbB*, 700 bp for *clbN*, and 821 bp for *clbQ*. 19 from 61 isolates were had *clbA*, *clbB*, *clbN* and *clbQ* island colibactin genes. Thus, the percentage of pks+ 31.14%, the percentage of pks– 68.85%. The pks– isolates varied in the genes they contained, as some genes were present and others were absent, while some isolates did not contain any genes. The results showed that the number of infected women was greater than the number of infected men, and the ages of infected women ranged between 35 and 77 years, while the ages of infected men were between 31 and 86 years. *P. alcalifaciens* is among pks+ bacteria carrying the four colibactin island genes (*clbA*, *clbB*, *clbN* and *clbQ*), as shown in Figure 3.

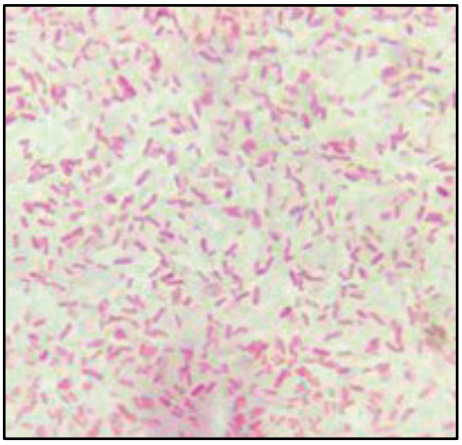

Fig 1. *Providencia alcalifaciens*: gram negative bacteria

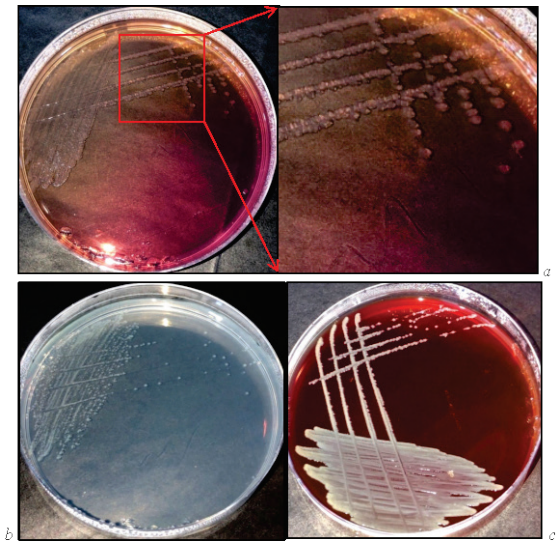

Fig. 2. *Providencia alcalifaciens*: *a* – MacConkey agar; *b* – nutrient agar; *c* – blood agar

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

Gene	Primer	Target, bp
clbA-F	CTAGATTATCCGTGGCGATTC	1002
clbA-R	CAGATACACAGATACCATTCA	
clbB-F	GATTTGGATACTGGCGATAACCG	550
clbB-R	CCATTTCCCGTTTGAGCACAC	
clbN-F	GTTTTGCTCGCCAGATAGTCATTC	700
clbN-R	CAGTTCGGGTATGTGTGGAAGG	
clbQ-F	CTTGTATAGTTACACAACTATTTC	821
clbQ R	TTATCCTGTTAGCTTTCGTTC	

Discussion

According to reports, microbes may be responsible for around 20% of human cancer cases, despite the fact that cancer is widely acknowledged to be a disease brought on by hereditary traits and environmental circumstances. Approximately one-third of tumors that cause death are digestive tract cancers. Infectious pathogens cause at least 15% to 20% of malignancies (Ağagündüz et al., 2023).

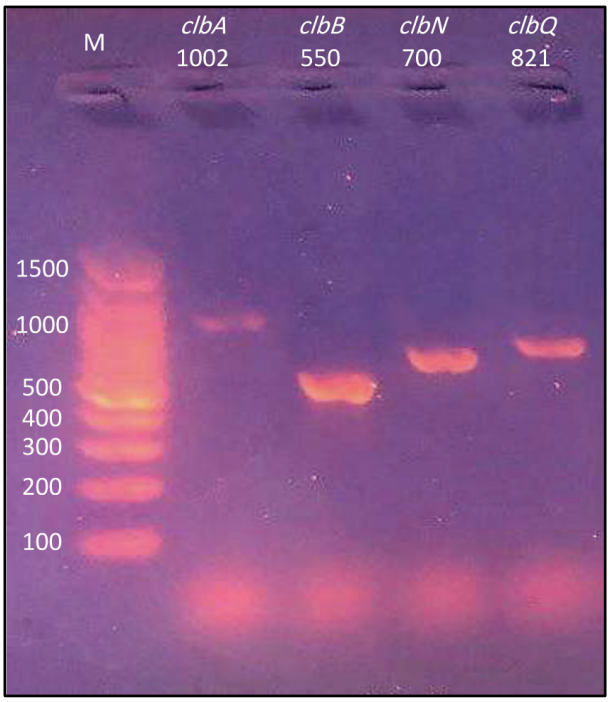

Fig. 3. Amplicons of *clbA*, *clbB*, *clbN*, and *clbQ* on 2% agarose gel from *Providencia alcalifaciens*

Numerous multi domain enzymes that are involved in the synthesis of the genotoxin colibactin are encoded by the pks gene cluster. These enzymes are commonly involved in the synthesis of secondary metabolites in a variety of microbial species. It has been demonstrated that this genotoxin is crucial in causing host cell DNA damage and breaks. Along with cell damage and heightened virulence in bacteria, colibactin may also play a role in the development of many disease entities. The gastrointestinal tracts of numerous mammals, including nearly all humans, include a member of the Enterobacteriaceae family. The promotion of colorectal tumor formation in vitro has been linked to the presence of the pks gene in some pathogenic and nonpathogenic *E. coli*. According to certain research, the intestinal mucosa of CRC patients had a higher expression of the pks gene than that of non-CRC controls (Hussein et al., 2020).

The species *P. alcalifaciens* belongs to the Enterobacteriaceae family's *Providencia* genus. As a negative for lactose test (nonfermenting bacterium), it forms pale colonies on enteric agars like *Salmonella-Shigella* agar, MacConkey agar, and desoxycholate citrate agar, just like other lactose nonfermenting bacteria like *Shigella*, *Proteus*, and *Salmonella*. Senior's 1997 study confirmed this (Bulach et al., 2024).

Providencia alcalifaciens is the most virulent *Providencia* species (Salas et al., 2023), the most prevalent in clinical isolates, and regarded as an opportunistic bacterial pathogen of humans. It causes a variety of nosocomial and environmental acquired diseases, such as meningitis, sepsis, diarrhea, urinary tract, wound, and ocular infections. Generally speaking, *Providencia* species are resistant to tetracyclines, aminoglycosides, penicillins, first-generation cephalosporins, and polymyxins (Guan et al., 2022). A significant public health concern is the rise of antibiotic resistance (Boattini et al., 2024; Sheet et al., 2024).

According to Putze a genomic island encoding the biosynthesis and secretion pathway of putative hybrid nonribosomal peptide polyketide colibactin has been described in Enterobacteriaceae such as *Escherichia coli*, *Klebsiella pneumoniae*, *Citrobacter koseri*, *Enterobacter aerogenes*, and isolates but not in *Providencia* (Putze et al., 2009). Our study is the first globally and locally to prove that *P. alcalifaciens* possesses the genomic island that is responsible for the production of colibactin toxin which plays a significant role in colon cancer, this. This is consistent with another study when proved that *Providencia* was elevated in the tumor microbiome, which further supports the idea that *Providencia* has an important role in colon cancer (Burns et al., 2015).

Conclusions

The relationship between *P. alcalifaciens* and colibactin genes highlights the potential pathogenicity of this bacterium, particularly in gastrointestinal contexts. While *P. alcalifaciens* has been recognized as an opportunistic pathogen, its genetic makeup suggests a capacity for virulence similar to that of other Enterobacteriaceae, including the presence of colibactin-related genes.

When *P. alcalifaciens* shows potential for pathogenicity, its role in human disease remains less understood compared to more established pathogens like *E. coli*. Further research is needed to clarify its clinical significance and the implications of colibactin gene presence.

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