Detection of *clbB* and *clbN* Genes in Isolated *Klebsiella Pneumoniae* of Biopsies from Colorectal Cancer Patient in Erbil Province

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Doi: 10.23918/eajse.v9i1p280

Abstract: Recently, it has been proved that some of Enterobacteriaceae like *Klebsiella pneumoniae*, which carry polyketide synthase (PKS) islands, damage human dsDNA by encoding Colibactin genotoxin; and finally, they induce some apoptosis in damaged mucosal cells. This study aimed to isolate the *clbN* and *clbB* genes, which are the markers of the PKS genomic island, from PKS cluster in these bacteria.

In this study, 100 biopsies were obtained, 50 from them from colorectal cancer patients and another 50 from normal patients as control from PAR International Hospital. Then, all samples were cultured on MacConkey agar medium and blood agar medium for bacterial isolation then, *Klebsiella pneumoniae* was identified by some biochemical testes (indole test, Cimon citrate test, urease test) and Vitek technique. Finally, The DNA of bacteria was extracted and then were amplified by PCR with specific *clbB and clbN* gene primers. We obtained (8) isolates out of 50 biopsies from patients with colorectal cancer; and 2 isolates *Klebsiella pneumoniae* out of 50 non-colorectal cancer patients as control. Further, all (8) *Klebsiella pneumoniae* that we obtained from colorectal cancer patients had both *clbB* and clbN genes. Whereas 2 *Klebsiella pneumoniae* that obtained from non-colorectal patients had just *clbB* and they did not have clbN. In this investigation conducted in Erbil province, *Klebsiella pneumoniae* with pks+ island was considerably greater in CRC patients compared to non-cancer individuals.

Keywords*:* Colorectal Cancer, Klebsiella *pneumoniae*, pks+ island, *clbB*, clbN, PCR

1. Introduction

Colorectal cancer is the second most common in women and third most common in men worldwide (Hama & Karim, 2019). There are some risk factors for this disease such as genetic background and environmental factors, as well as bacterial infections (Giovannucci and Willett, 1994). *Klebsiella pneumoniae* is a Gram-negative immobile, encapsulated, lactose fermenting, facultative anaerobic, and rod-shaped bacteria (Candan and Aksöz, 2015).

The *polyketide synthase(pks)* genotoxic *K. pneumoniae* has recently triggered a widespread alarm. DNA damage and higher virulence have been linked to colibactin, a genotoxin expressed by the *pks* genomic island (Mohandas, 2011). The *pks* island contains 19 colibactin synthesis genes (*clbA-clbR*); only the presence of all *pks* genes is required for complete colibactin production (Nougayrède et al., 2006). *clbB* (9629 bp) and *clbN* (4372 bp) are as molecular markers respectively for spaces of 3' and 5'. These two genes associated with each other create a pre-reconstruction named Nacyl- D-asparagine which finally produces Colibactin according to the effect of *clbP*. On the other hand, *clbQ* and *clbA* as the final markers are subset to the *PKS* genomic island (Brotherton and Balskus, 2013).

Received: December 17, 2022
Accepted: January 20, 2023
Ibrahim, J., & Salah, H.F. (2023). Detection of clbB and clbN Genes in Isolated *Klebsiella pneumoniae* of Biopsies from Colorectal Cancer Patient in Erbil Province. *Eurasian Journal of Science and Engineering, 9*(1), 280-290.

The pks island is 54 kb in size and has been extensively characterized in numerous Enterobacteriaceae members. (Chen et al., 2017).

Then studies showed that a certain type of infection of *Enterobacteriaceae* especially *E. coli* and *Klebsiella pneumoniae* with PKS has a positive strain. This strain of bacteria can promote colorectal cancer in patients with inflammatory bowel disease (IBD), Crohn's disease, and ulcerative colitis (Tomkovich and Jobin, 2018). The bacteria with PKS genes (genome cluster) can produce a kind of exo-toxin colibactin which is able to damage the host genomic cells subjected to compose inflammation all over the colon's wall. A kind of genomic cluster (54 kbp) has been placed in asnW tRNA locus, liable to Colibactin synthesis. (Wernke et al., 2020)

The Colibactin damages double stranded DNA and increased level of H2AX histone, lead to the activation of the DNA damage response (DDRs). DDRs are necessary for protection of genetic information and the identification of cell genome against the invasion of environmental factors and other ROS endogenous activities. This mechanism along a molecular cascade causes the stop of cell cycle through G2 process. If the restoration rose in a normally way, the cell proliferation has to become in a natural circle; otherwise, the cell deviates to the apoptosis in which mutation may come to be happened in the genome (Kurokawa et al., 2007).

So, it will lead to the initiation and progression of colorectal cancer by considering this fact that the bacteria with PKS gene disrupt the cell cycle by producing the colibactin as secondary metabolites of bacteria. Therefore, the aim of this paper is to detection of *clbB* and *clbN* genes in *Klebsiella pneumoniae* of biopsies from patients with colorectal cancer in Erbil province – Kurdistan region of Iraq.

2. Materials and Method

2.1 Specimens' Collection

In this study, as a cross–sectional and experimental study, we collected 100 biopsies, 50 from them from colorectal cancer patients and another 50 from non-colorectal cancer patients as control, referred to the Par International Hospital., for 8 months. In addition, this study was approved by Ethics Committee of Salaheddin university, with reference No:4438/2/3. The samples were placed into the sterilized tub that contains phosphate buffer (pbs). Then, immediately transferred into the Molecular Laboratory in Department of Biology college of education in Salaheddin university. Then were centrifuged the samples by 7000 RPM and 5 minutes.

2.2 Bacteriological Examination

To isolate the *Klebsiella pneumoniae* the samples were cultured using blood and MacConkey agar media respectively. After that, various biochemical tests such as the indole, citrate utilization, and urease tests were utilized to future identification. Finally, the presence of *Klebsiella pneumoniae* was confirmed using the Vitek technique.

2.3 Molecular Examination and Primer Design.

The DNA of isolates were extracted using AccuPrep Genomic DNA Extraction kit (Republic of Korea).valuation of quality and quantity of extracted DNAs were done by electrophoresis on 1 % agarose gel and nanodrop on 260 and 280 nm respectively.

The sequence of designed specific primers for clbB and clbN genes are listed in table (1).

Table 1: The utilized primer sequences, PCR product.

Genes	*Primer name*	Primer sequence (5' to 3')	Size of product	Reference
clbB	*ClbB* F	5'-GACAGGCTATGCTACCGCCA -3'.	175 bp	This study
	ClbB R	5'-CATCAATGGGCGCAGTCCAC-3'.	//	
clbN	*ClbN* F	5'-GCTACCGCTGCATTTCCCAC-3'.	160 bp	
	ClbN R	5'-CGCCAATGCCGTTAGCACAT-3'.	//	

Furthermore, we optimized the annealing temperature and concentration of primers under study in the purpose of getting a good result.

2.4 Polymerase chain reaction (PCR)

The PCR carried out as 1 µ1 or 100 ng DNA, 12 µ1 Master mix (Ampliqon Co. Denmark), 1µ1 of each primer (forward and reverse) and 10 µ1 sterilized deionized water with 25 µl total volume for each reaction. The thermocycler (Techno, UK) used as 32 cycles with 95ºC denaturation, 59ºC annealing and 72ºC extension respectively. and final extension temperature was 72ºC for 7 minutes. After that, all of the PCR products were analyzed by electrophoresis on 1% agarose gel.

2.5 Statistical Analysis

Statistical analyses were performed using the GraphPad prism program, version 8, Fisher exact test and the Chi-square test. Moreover, if 0.05, the p-value was deemed important.

3. Results

3.1 Biochemicals and Vitek II Technique

In this study, we could isolate 10 *Klebsiella pneumoniae* out of 100 biopsies. Amongst, 8 (16%) from patients with colorectal cancer and 2 (4%) were isolated out of 50 normal persons that we took them as a control. The *K. pneumoniae* isolates were confirmed through both biochemical testes and vitikII technique. Consequently, Once the bacteria were cultured on MacConkey agar, we utilized various biochemical tests (indole test, citrate utilization test, and urease test) to identify them. Out of the cancer samples tested, we identified 12 samples as *Klebsiella pneumoniae* based on the results of the biochemical tests: negative for indole test, positive for citrate test, and positive for urease test, as indicated in the table (2).

Table 2: the biochemical tests for bacteria isolated from CRC patients

isolates	Indole	Citate	urease
1	-	-	-
2	+	-	-
3	+	-	-
4	-	+	+
5	-	-	-
6	+	-	-
7	+	-	-
8	-	+	-
9	-	-	+
10	-	+	+
11	+	-	+
12	-	+	+
13	+	-	-
14	-	+	+
15	+	-	-
16	+	-	-
17	+	-	-
18	+	-	-
19	+	-	-
20	-	+	+
21	+	+	+
22	+	+	-
23	+	+	-
24	+	+	-
25	-	+	+
26	-	+	-
27	-	-	+
28	+	+	-
29	-	-	+
30	+	-	-
31	+	-	-
32	-	-	-
33	+	-	-
34	+	-	-
35	+	-	-
36	+	-	-
37	-	+	+
38	-	+	+
39	-	+	+
40	-	+	+
41	-	-	+
42	+	-	-
43	-	+	+
44	+	-	-
45	-	+	+
46	+	-	-
47	+	-	-
48	+	-	-

49	-	+	+
50	-	-	-

For more emphasis in identification the biochemical tests for cancer samples, we did Vitek II for 12 samples that were their biochemical tests are negative for indole, positive for citrate and positive for urease we got 8 isolates klebsella pneumoniae as shown in the (table 3).

Table 3: the result of Vitek II techniques for bacteria isolated from CRC patients.

isolates	Vitek results
12	Klebsella pneumoniae
14	E coli
20	E coli
21	E coli
25	Klebsella pneumoniae
29	E coli
37	Klebsella pneumoniae
39	Klebsella pneumoniae
40	Klebsella pneumoniae
43	Klebsella pneumoniae
45	Klebsella pneumoniae
49	Klebsella pneumoniae

In regards the non -cancer samples, the 5 samples were indole negative, citrate test positive, and urease test positive that refer to the *Klebsiella pneumoniae* as shown in the (4 table).

Table 4: the biochemical tests for bacteria isolated from non-CRC patients

isolates	Indole	Citate	urease
1	+	-	-
2	+	-	-
3	+	-	-
4	-	+	+
5	+	+	+
6	-	+	+
7	-	+	+
8	+	-	-
9	-	-	+
10	-	-	+
11	+	-	-
12	-	+	+
13	+	-	-
14	-	-	-
15	-	+	+
16	-	+	+
17	+	-	-

18	-	+	+
19	+	-	-
20	+	-	-
21	+	-	-
22	+	-	-
23	+	-	-
24	+	-	-
25	+	-	-
26	+	-	-
27	+	-	-
28	+	-	-
29	+	-	
30	+	+	+
31	+	-	-
32	+	_	-
33	+	-	-
34	+	-	-
35	+	+	-
36	-	+	-
37	-	-	-
38	-	-	+
39	-	+	-
40	+	-	-
41	+	-	-
42	+	+	-
43	+	-	
44	+	+	+
45	+	-	-
46	+	-	-
47	+	-	-
48	-	-	-
49	-	+	+
50	-	-	+

In regards non-cancerous samples, the Vitek II was performed for 5 samples that were indole negative, citrate positive and positive for urease we got 2 isolates *Klebsiella pneumoniae* (Table 5).

Table 5: the result of Vitek II techniques for bacteria isolated from non-CRC patients.

isolates	Vitek results
4	Raoultella Planticola
6	E coli
7	Klebsella pneumoniae
30	Enterobacter aerogenes
48	Klebsella pneumoniae

3.2 Molecular Based Technique

Following the biochemical examination, the conventional PCR analysis were performed for 10 isolates *Klebsiella pneumoniae* that were taken in both cancer and non-cancer samples. The results showed that the frequency of *Klebsiella pneumoniae* with clbB positive strains was 100%. It means that 8 samples out of 8 were positive for clbB (Fig 1).

Moreover, the frequencies of *Klebsiella pneumoniae* with clbN positive strains was (100%) (Fig 2). Means in colorectal patients all isolates *K. pneumoniae* had both clbB and clbN. The analysis also showed that 8 samples with 100% frequency were positive for both clbB and clbN genes as simultaneous from patients with colorectal cancer, but in normal patients that we used as a control from 50 biopsies just we isolated 2 *Klebsiella pneumoniae* so, the results showed that the frequency of *Klebsiella pneumoniae* with clbB positive strains in normal person was 100 %. As shown in (fig 1) It means those samples are positive for clbB but the frequency of *Klebsiella pneumoniae* with clbN positive strains in normal person was 0 % as shown in (fig 2) means those two samples are negative for clbN, as shown in table (6). Moreover, after chi square analysis calculation p value found to be less than 0.05 which indicates that *Klebsiella pneumoniae* that have both genes significantly affects colorectal cancer.

Table 6: Calculation of P value between genes positive and negative *K. pneumonia* species with CRC and normal population

Data analyzed	Cancer	Normal	Total
ClbB and *clbN* positive	8	0	8
clbB and *clbN* negative	42	50	92
Total	50	50	100
P value = 0.0058 so it is less than 0,05 and it is significant			

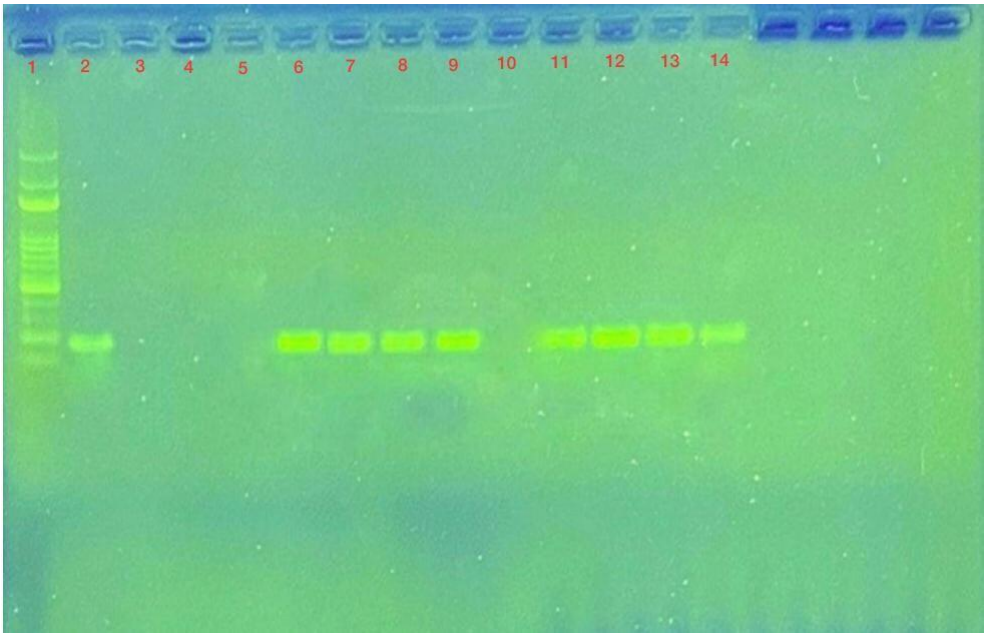

Figure 1: Analysis of *clb*N gene on 1% agarose gel. From left to right; lane1, ladder 100bp, Fermentase Co. Lane 2: Positive control, brought from Erbil. lane 3: Negative control brought from media center in Erbil. lane4: negative control (primer f and r, master mix, water without template) lanes 5-13: PCR products of all samples with *clb*N gene. The size of PCR product for *clb*N gene is 160 bp.

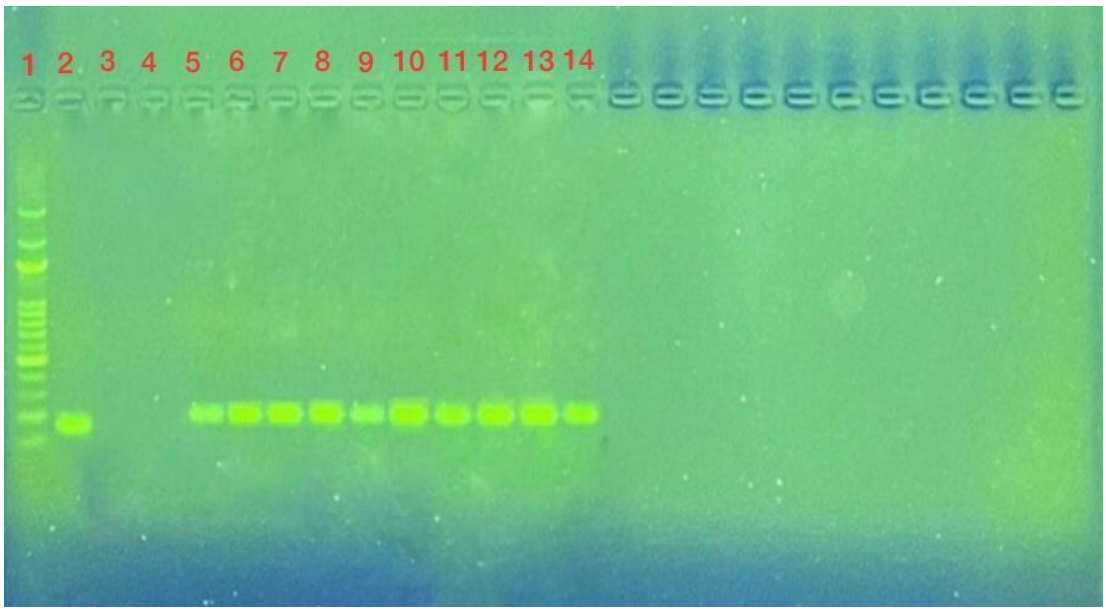

Figure 2: Analysis of *clbB* gene on 1% agarose gel. From left to right; lane1: ladder 100bp, Fermentase Co. Lane 2: Positive control, bought from Erbil. lane 3: Negative control bought from Media center in Erbil. lane 4: negative control (primer f and r, master mix, water without template). lanes 5-13: PCR products of all samples with *clbB* gene. The size of PCR product for *clbB* gene is 175 bp.

4. Discussion

As it was shown above in our study 8 (16%) Klebsiella *pneumoniae* isolated out of 50 biopsies from patients with colorectal cancer and 2 (4%) Klebsiella *pneumoniae* isolated out of 50 normal persons that we took them as a control (non-CRC). However, the number of isolated *Klebsiella pneumoniae* in colorectal patients more than the non-colorectal patients, the Chi square result showed (P value=0.0916) which is more than (0.05) which means there is nosignificant relation between *Klebsiella pneumoniae* with colorectal cancer. Otherwise, all people wouldhave developed CRC at some point in their lives because. according to a research conducted in Cambridge University the genus Klebsiella is the second most populous enteric genus found in the gastrointestinal tract of man (Ristuccia and Cunha, 1984). Furthermore, in our study sample from CRC patients whom *K. pneumonia* isolated, contained both clbB and clbN genes. However, the two patientswhich did not have CRC in which *K. Pneumonia* were isolated only have clbB gene and did not have clbN. The Chi square analysis showed (P value=0.0058) which is less than (0.05) which means there is a significant relation between *Klebsiella pneumoniae* that has both genes with colorectal cancer. This indicates the necessity of clbB gene to clbN gene for production of colibactin. The finding of the current research can be supported with other researchers conducted in the United Kingdom, France, and Malaysia found that pks+ including clbB and clbN *Klebsiella pneumoniae* was considerably greater in CRC patients compared to non-cancer individuals (Buc et al., 2013). However, in a Japanese population no significant difference found in the proportion of pks+ including (clbB and clbN) genes between CRC patients and healthy controls (Shimpoh et al., 2017). It is hypothesized that microbiota composition varies by geographic region, which might explain the disparity in the distribution of the pks island in the Philippines, Malaysia, Japan, Europe, UK and France (Lozupone et al., 2012).

Furthermore, the different types of tissue samples used for molecular analysis might have influenced the results. Fresh biopsy tissues were employed in both the UK and France trials; Malaysia used in vitro tests; and Japan used colonic lavage and FFPE colorectal tissues, respectively (Arthur et al., 2012). Putze, et al (2009) have done their investigation over genetic structure of PKS and genetic distribution between other Enterobacteriaceae bacteria, the results of mentioned study have indicated that among 1565 isolated samples, 3.5% with *Klebsiella pneumonia*, in which all of them were clbB and clbN positive. Compare to our results in which 16% of CRC patients were klebsiella positive, and all of them contained both genes. This shows a strong similarityin which 100% of patients with *K. pneumonia* contained clbB and clbN genes together. The current study tried to analyze the involvement of clbB and clbN genes in the PKS Island, but this time in Erbil. Unlike other studies only *Klebsiella pneumonia* was isolated from colorectal cancer. The positive control strain employed in this testing was *Klebsiella pneumonia* strain, which is also the name of its detector, which was purchased here by Erbil Media Center. A study to show significance of presence of K. Pneumoniae with *clbB* and *clbN* genes in normalpopulation to the development of CRC in future, for this purpose of using these two genes as a biomarker screening program for highly risky people. So that early measures could be taken forprevention of CRC.

5. Conclusion

In conclusion, in this study we tried to explain the relationship between *K. pneumoniae,* and CRC and it was found that there is no significant relationship. Moreover, we tried to analyze those *K. pneumoniae* bacteria found in CRC patients' biopsies we got the result that both clbB and clbN genes must be positive for CRC to develop. Finally, we suggest that this study be used as a framework for future studies to develop a screening program for CRC in the future. We also recommend future study with higher sample size.

6. Acknowledgments

The authors should thank the colonoscopy department in PAR international hospital and Dr Lukman Aziz, for collection of the samples, and my wife for supporting me also all friends that helped me during the research.

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