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Colonization of the gut mucosa of colorectal cancer patients by pathogenic mucosa-associated *Escherichia coli* strains

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ABSTRACT

Some strains of *Escherichia coli* are known to be involved in the pathogenesis of colorectal cancer (CRC). The aim of current study was to compare the general characteristics of the *E. coli* from CRC patients and healthy participants. A total of 96 biopsy samples from 48 CRC patients and 48 healthy participants, were studied. The clonality of the *E. coli* isolates was analyzed by Enterobacterial repetitive intergenic consensus-based PCR (ERIC-PCR) method. The strains were tested by PCR to determine the prevalence of different virulence factors. According to the results of ERIC-PCR analysis, (from the 860 *E. coli* isolates) 60 strains from CRC patients and 41 strains from healthy controls were identified. Interestingly, the majority of the strains of both groups were in the same cluster. Enteropathogenic *E. coli* (EPEC) was detected significantly more often in CRC patients (21.6 %) than in healthy participants (2.4 %) ($p < 0.05$). The Enteroaggregative *E. coli* (EAEC) was found in 18.33 % of the strains of CRC patients. However, other pathotypes were not found in the *E. coli* strains of both groups. Furthermore, all the studied genes encoding for virulence factors seemed to be more prevalent in the strains belonging to CRC patients. Among the virulence genes, the statistical difference regarding the frequency of *fuyA, chuA, vat, papC, hlyA* and *cnf1* genes was found significant ($p < 0.05$). In conclusion, *E. coli* strains that carry extraintestinal pathogenic *E. coli* (ExPEC) and diarrheagenic *E. coli* (DEC) multiple virulence factors colonize the gut mucosa of CRC patients.

1. Introduction

Colorectal cancer (CRC) is the third most commonly diagnosed cancer and the second most deadly cancer in the world [1]. CRC is a complex disease with multiple risk factors. The most important risk factors for this type of cancer are race and ethnicity, age, hereditary mutations, inflammatory bowel disease (IBD), obesity, smoking, alcohol consumption and diabetes[2].

Recently literature suggests that intestinal microbiota could be associated with colorectal carcinogenesis [3–6]. The human gut microbiota contains a collection of various microbes such as bacteria, fungi, viruses and archaea that live in the normal intestinal tract [7,8]. Gut microbiota performs a vital role in bodily functions including metabolization of dietary compounds, maintaining the physiological functions of the intestinal tract and modulating the host immunity [7]. Various factors such as environment trigger, genetic defect and diet can change the composition of the gut microbiota, the so called microbial dysbiosis [9]. Microbiota dysbiosis has been recognized as a cause of various disorders like obesity, diabetes, allergies, CRC and IBD [10]. Levels of some bacteria such as *Prevotella, Bacteroides* spp. [11], *Escherichia coli, Bacteroides fragilis* and *Streptococcus gallolyticus* have been found to be higher in CRC biopsy specimens than in healthy tissues [12].

Given that *E. coli* is the most common human gut commensal facultative anaerobic resident [13], a significant number of studies have clearly shown that there is a clear link between specific strains of this bacterium and CRC [14–16]. Mucosa-associated *E. coli* especially cyclomodulin-positive strains are colonized more frequently in gut mucosa of patients with CRC in comparison to the normal mucosa [16].

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Mucosa-associated *E.coli* has an important role in the pathogenesis of CRC by stimulation of a pro-inflammatory response [17] and production of toxins [18]. It has also been observed that such *E.coli* strains from CRC patients carry specific virulence factors of the extra-intestinal pathogenic *E. coli* (ExPEC), especially uropathogenic *E. coli* (UPEC) such as pyelonephritis-associated pilus (P fimbriae or pap) Yersinia bactin receptor (fyuA), the heme receptor (chuA) and vacuolating autotransporter toxin (vat) [19]. More noteworthy, enteropathogenic *Escherichia coli* (EPEC) strains have been found more frequently in the tissue samples of CRC patients than those with healthy samples, so there may be a correlation between some strains of diarrheagenic *E. coli* (DEC) and CRC [20].

To our knowledge, little data are available on the general characteristics of the mucosa-associated *E. coli* strains from CRC patients. Therefore, this study was aimed to evaluate the genetic diversity, pathotypes and virulence gene profile of mucosa-associated *E. coli* from patients with CRC and healthy subjects.

2. Materials and methods

2.1. Specimen collection and processing

In a cross-sectional study, biopsy specimens were collected from 48 CRC patients and 48 from normal mucosa of healthy subjects who underwent colonoscopy for colon cancer screening at referral university-affiliated hospitals in northwest Iran from July 2019 to August 2020. Each lazed biopsy sample were directly cultivated on MacConkey agar [21]. *E. coli* isolates were identified by standard biochemical tests.

2.2. Enterobacterial repetitive intergenic consensus-based PCR (ERIC-PCR)

The clonality of the 860 *E.coli* isolates obtained from healthy subjects and CRC patients were typed using ERIC-PCR with the primer sequence "ATGTAACGTCCTGGGGATTCAC" [22] under the following conditions: denaturation (94 °C for 4 min), followed by 35 cycles of denaturation (94 °C for 30 sec), annealing (47 °C for 1 min) and extension (72 °C for 1 min) along with a final extension at 72 °C for 4 min. The electrophoresis results were analyzed using SPSS software. The dendrogram was produced using algidus interval method on the basis of centroid profile.

2.3. Determination of pathotypes

Pathotypes of mucosa-associated *E. coli* strains were determined by examining the presence of the following genes: *estA* and *eltB* for enterotoxigenic *E. coli* (ETEC), *eae* and *bfp*A for enteropathogenic *E. coli* (EPEC), *vt1* and *vt2* for enterohemorrhagic *E. coli* (EHEC), *ial* for Enteroinvasive *E. coli* (EIEC) and *pCVD432* for Enteroaggregative *E. coli* (EAEC).

In this study, the template DNA from the *E. coli* strains was extracted by DNA extraction kit (Sinaclon, Iran) according to the manufacturer's instructions. The PCR reaction was performed in a 50 μL mixture containing 1.0 U of DNA polymerase (Yekta Tajhiz Azma, Iran), 10–100 ng of the template DNA, 0.2 mM dNTPs, 3 mM MgCl2 and 0.5 pmol of each primer in the corresponding reaction buffer. The sequence of primers and PCR conditions have already been reported [21,23,24].

2.4. ExPEC related virulence factor-encoding genes detection

The prevalence of adhesin-encoding genes (*fimH, papC, afa/BC),* toxin-encoding genes *(hlyA, cnf1, vat)* iron transports (*chuA, fuyA)* and protectin/invasion-encoding gene (*ibeA)* was performed by PCR method. The sequence of primers and PCR conditions have already been described [21,25–28].

2.5. Statistical analysis

In the present study, the results were analyzed by the Chi-square test or Fisher's exact test using SPSS software for Windows (version 23 SPSS Inc.,Chicago, IL, USA). The statistical significance level was considered at P-value < 0.05

3. Results

3.1. Genotyping of E. coli isolated from CRC patients and healthy subjects

We examined 860 *E. coli* isolates from 48 CRC patients and 48 healthy subjects. The results of ERIC-PCR analysis yielded 60 *E. coli* strains from CRC patients (23 strains from proximal colon cancer and 37 strains from distal colon and rectal cancer) and 41 *E. coli* strains from control subjects. As a result, a total of 101 *E. coli* strains were taken into the study. Clustering based on fragment profiles genotyped the *E. coli* strains in 15 clusters (80 % similarity). The majority of *E. coli* strains from CRC patients (81.66 %) and control group (85.36 %) belonged to cluster I (Fig. 1). There was no significant difference in the distribution of strains obtained from the CRC patients and the control group within the identified clusters.

3.2. PCR-Based pathotyping

The results of the pathotyping revealed that 40 % (n = 24) of the *E. coli* strains from CRC patients were confirmed as pathogenic; 13 (21.6 %) were EPEC, 11 (18.33 %) were EAEC. Moreover, EPEC was found in 2.4 % of control strains. The increased frequency of EPEC strains in CRC patients compared to controls were statistically significant ($p < 0.05$). Other *E. coli* pathotypes were not found in the strains of both groups.

3.3. Prevalence of virulence genes of E. coli strains

E. coli strains from CRC patients and healthy subjects were screened for the presence of several genes encoding virulence factors. As shown in Fig. 2, the biopsy specimens of CRC patients, specifically distal colon and rectal cancers, were more colonized by mucosa-associated *E. coli* encoding virulence factor than those of the control samples.

In this study, the most prevalent virulence genes in *E.coli* strains from healthy subjects were *fimH* (92.68 %), *afaBC* (85.36 %), *ibeA* (68.92 %), *vat*(31.7 %), *chuA* (31.7 %), *fuyA* (29.26 %), *papC* (14.28 %) and *eae* (2.4 %). The *hlyA, cnf1* and *pCVD432* genes were not found in strains from the control subjects. Moreover the most prevalent virulence genes in *E. coli* strains from CRC patients were *fimH* (95 %), *afaBC* (86.66 %), *fuyA* (80 %), *chuA* (78.33 %), *ibeA* (76.66 %), *vat* (60 %), *papC* (33.33 %), *hlyA* (30 %), *eae* (21.6 %), *pCVD432* (18.33 %) and *cnf1(*13.3 %). Noticeably, there was a significant difference in the prevalence of, *fuyA, chuA, vat, papC, hlyA, eaa, pCVD432* and *cnf1* genes between the *E.coli* strains from CRC patient and healthy subjects. Furthermore, the increased frequencies of *fuyA, chuA, vat, hlyA* and *pCVD432* genes on distal colon and rectal cancer samples were statistically significant as compared to proximal colon cancer tissues ($p < 0.05$) (Fig. 2).

3.4. Prevalence of ExPEC related virulence factors in DEC strains

In this study, the *fimH, afa/BC, vat, fuyA, chuA, ibeA, papC, hlyA* and *cnf1* genes were detected in 92.3 %, 92.3 %, 76.9 %, 76.9 %, 69.2 %, 69.2 %, 69.2 %, 46.1 % and 30.7 % of the EPEC strains belonged to CRC patients, respectively. Moreover, all the EPEC strains from CRC patients carried more than one virulence factor, so that, 23 %, 30 %, 23 %, 7.7 %, 7.7 % and 7.7 % of the EPEC strains simultaneously carried 8, 7, 6, 5, 3 and 2 genes related to ExPEC, respectively (Table 1). In addition, the EPEC strain belonged to the control group had *fimH* and *afa/BC* virulence genes.

Among EAEC strains, *fimH, afa/BC, vat, fuyA, ibeA, chuA, papC, hlyA*

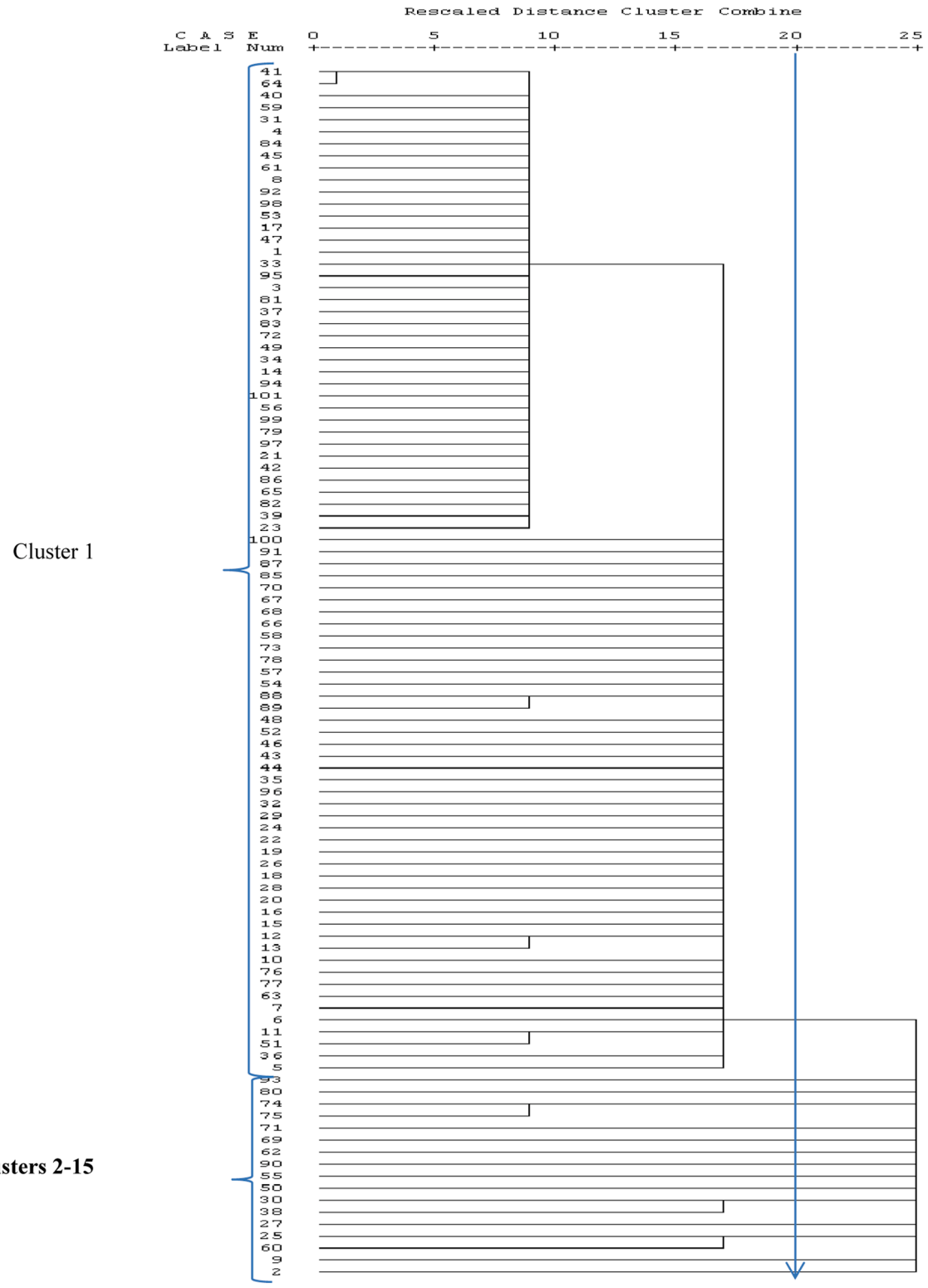

Fig. 1. Dendrogram based on ERIC-PCR pattern analysis of 60 mucosa-associated *E.coli* strains from CRC patients and 41 strains from healthy controls.

and *cnf1* genes were observed in 100 %, 81 %, 63.6 %, 72 %, 63.6 %, 72 %, 36.3 %, 27 % and 18.18 % of the strains, respectively. Moreover, 18.18 %, 18.18 %, 9 %, 27.27 %, 9 % and 18.18 % of the EAEC strains simultaneously carried 8, 6, 5, 4, 3 and 2 genes related ExPEC, respectively. There was no significant difference in the prevalence of virulence-encoding genes in EPEC and EAEC strains from CRC patients ($P > 0.05$) (Table 1).

4. Discussion

Recently published literature indicated that intestinal microbiota could be associated with colorectal carcinogenesis [3,4]. To our knowledge, there is little data regarding the characteristics of carcinogenic strains. Unlike previous studies that focusing more on the prevalence of cyclomodeline toxins, we examined different virulence factors

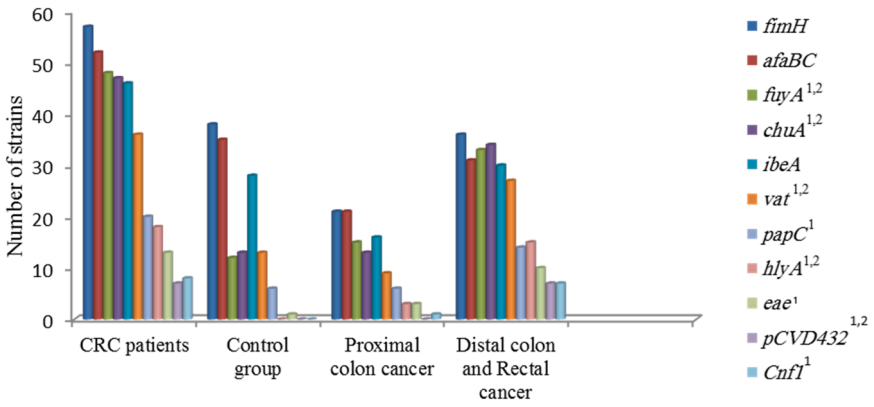

Fig. 2. Distribution of genes encoding virulence factors according to specimen origins
[1], The increased frequencies of virulence-encoding genes in *E. coli* strains isolated from CRC patients compared to control tissues were statistically significant ($p < 0.05$);
[2], The increased frequencies of virulence-encoding genes in *E. coli* strains isolated from distal colon and rectal cancer compared to proximal colon cancer tissues were statistically significant ($p < 0.05$).

Table 1
Prevalence of several genes- encoding virulence factors in EPEC and EAEC strains.

Number(percentage) of *E. coli* strains exhibiting virulance-encoding genes				
Category	Gene	EPEC (n=13)	EAEC(n=11)	*P* value
Adhesins	*fimH*	12(92.3)	11(100)	$P > 0.05$
	afa/BC	12(92.3)	9(81)	$P > 0.05$
	papC	9 (69.2)	4 (36.3)	$P > 0.05$
Toxins	*vat*	10(76.9)	7(63.6)	$P > 0.05$
	hlyA	6(46.1)	3(27)	$P > 0.05$
	cnf1	4(30.7)	2(18.1)	$P > 0.05$
Iron transports	*fuyA*	10(76.9)	8(72)	$P > 0.05$
	chuA	9(69.2)	8(72)	$P > 0.05$
protectin/invasion	*ibeA*	9 (69.2)	7(63.6)	$P > 0.05$

among *E. coli* strains from CRC patients and healthy individuals.

In the present study, 60 mucosa-associated *E. coli* strains from patients with CRC and 41 *E. coli* strains from the healthy subjects were identified. Buc et al [16], Bonnet et al [14], and Martin et al [21] already reported higher numbers of mucosa-associated *E. coli* strains in biopsy samples of patients with CRC as compared with the normal tissues.

The analysis of similarity level, among the mucosa-associated *E. coli* strains, revealed that strains from CRC patients possessed a high genetic relatedness with strains obtained from the control group samples. Most of the strains isolated from the two groups were in the same cluster. Since ERIC-PCR genotyping does not indicate a significant connection with any parameter, it can be said that phenotypic features might be more crucial factors in separating the two groups' strains.

Examination of biopsy specimens of patients with CRC and healthy individuals showed that CRC samples are significantly more colonized by DEC strains such as EPEC (21.6 %) and EAEC (18.3 %) in comparison to the healthy subjects. Therefore, these strains could be involved in the pathogenesis of CRC. Magdy et al, reported the prevalence of EPEC in 50 % and 20 % of strains isolated from CRC patients and the control group, respectively [20].

In the present study, biopsy specimens of CRC patients specifically distal colon cancers were significantly more frequently colonized by mucosa- associated *E. coli* encoding virulence factors than the control samples, suggesting that distal colon tissue provides a selective condition for the colonization of these strains. Buc et al. have previously reported the distal colon cancer tissues more colonized by cyclomodulin-producing *E. coli* strains than the proximal colon cancers or control specimens [16]. Higher presence of pathogenic *E. coli* strains in gut mucosa of CRC patients compared to healthy controls can be explained that changes in the set of tumor tissue receptors can increase the colonization of these bacteria in the tumor microenvironment [16].

In the current research, most strains isolated from CRC patients possessed at least two specific virulence factors of ExPEC such as *fyuA, chuA, papC, hlyA, cnf1* and *vat*. Bronowski et al. detected UPEC-associated virulence genes among the mucosa-associated *E. coli* isolated from patients with CRC. They concluded that the presence of such strains in the gastrointestinal tract can trigger cellular proliferation of intestinal epithelial cells [19].

In this study, the most commonly detected virulence genes in CRC patients and healthy subjects were *fimH* (95 % in the CRC patients and 92.6 % in the control group) and *afaBC* (86.6 % in the CRC patients and 85.3 % in the control subjects). No significant difference was observed in the prevalence of mentioned genes between the strains of CRC or control patients. However, the expression level of *fimH* and *afaBC* genes may be different in the two groups. Carcinoembryonic antigen related cell adhesion molecule 6 (CEACAM6) is a tumor marker, whose increased expression has been detected in colorectal tumors [16]. Adherent-Invasive *E. coli* strains could adhere to and invade epithelial cells through FimH pili by binding to CEACAM6 [29]. Therefore, this type of pili may be an important virulence factor for mucosa- associated *E. coli* strains in the pathogenesis of CRC. Noticeably, among the adhesin-related genes, only the frequency of *papC* gene was significantly higher in cancer patients as compared to the control subjects. PapC adhesin has been recognized as characteristic extraintestinal virulence factor that is involved in the initiation of infection [30].

In this study, high prevalence of *fyuA* (80 %) and *chuA* (78.3 %) genes was detected in mucosa-associated strains from CRC patients as compared to the control patients ($p < 0.05$). ChuA and FyuA proteins are essential for iron uptake [31] they can, therefore, play an important role in bacterial colonization and persistence in the tumor microenvironments. Zareie et al. observed *chuA* gene in 42 % of strains from patients with CRC and 47 % of strains from the participants in the control group [32]. Furthermore, in the current study, the prevalence of genes encoding for HlyA, Cnf1 and Vat toxins was found to be significantly higher than that of the samples in the control group ($p < 0.05$). HlyA toxin impairs colonic epithelial barrier function, resulting in increased antigen uptake and initiating intestinal inflammation. Therefore, HlyA-producing strains of *E.coli* can be considered as a cofactor in the pathogenesis of intestinal inflammation [33]. It should be noted that the inflammation is one of the important risk factors for CRC [34]. The CNF1 toxin can lead to cytoskeletal alterations, affecting the cell cycle. Therefore, it can be involved in cancer by causing chromosomal instability [35,36]. Buc et al. detected the presence of *cnf* gene in about 39 % of CRC biopsy samples and 12 % of diverticulosis [16]. Vat is a serine protease autotransporter protein. This toxin may play a pivotal role in

the pathogenesis of IBD by inducing severe inflammation [37]. Since IBD is an important risk factor for CRC ([38], it can also be involved in the development of CRC.

Interestingly in this study, all of the EPEC and EAEC strains carried at least two specific virulence factors of ExPEC strains. These hybrid ExPEC/DEC *E. coli* strains were found in the biopsy specimens of CRC patients with a significantly higher frequency in comparison to those in the control subjects. However, the data on the frequency of hybrid ExPEC/ DEC strains in CRC patients seem to be insufficient. Indeed, whether and how these strains influence the pathogenicity of CRC is a question whose answer requires more research in a longitudinal observational study.

Thus, we suggest future research investigate the level of expression of virulence genes related ExPEC strains isolated from CRC patients control patients. Additionally, the effect of mucosa-associated *E. coli* strains obtained from CRC patients, especially ExPEC/DEC strains, on cell lines and animal models should be studied in order to accurately determine whether these strains are involved in colorectal carcinogenesis.

5. Conclusion

Our study showed that mucosa-associated *E.coli* that carry ExPEC/ DEC multiple virulence factor, including pili adhesins, toxins and iron capture systems are colonized more frequently in the gut mucosa of patients with CRC than in healthy individuals. To summarize, some mucosa-associated *E.coli* strains obtained from CRC patients, could play a role in colorectal carcinogenesis, but more studies and research is required to confirm the carcinogenesis potential of this strains. To the best our knowledge this is the first report of hybrid ExPEC/ EPEC and ExPEC/ EAEC strains from patients with CRC from Iran.

CRediT authorship contribution statement

Roghayeh Nouri: Data curation, Investigation, Methodology, Project administration, Visualization, Writing – original draft, Writing – review & editing. **Alka Hasani:** Conceptualization, Data curation, Software, Writing – review & editing. **Kourosh Masnadi Shirazi:** Data curation, Investigation, Methodology. **Fatemeh Yeganeh Sefiadn:** Data curation, Formal analysis. **Fariba Naeimi Mazraeh:** Data curation, Formal analysis. **Simin Sattarpour:** Data curation, Writing – review & editing. **Mohammad Ahangarzadeh Rezaee:** Conceptualization, Supervision, Validation, Visualization, Writing – review & editing.

Declaration of competing interest

The authors declare no conflict of interest

Ethical approval

This study was approved by the research ethics committee (IR. TBZMED. REC.1398.409) at Tabriz University of Medical Sciences, Tabriz, Iran.

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Author agreement statement

We confirm that this manuscript neither the entire paper nor any part of its content has been published or being considered for publication elsewhere. All authors have read and approved this manuscript and take responsibility for its contents.

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