



Evaluation of Fecal Colonization with Extended-Spectrum Beta-Lactamase-Producing Enterobacteriaceae and Risk Factors among Cancer Patients in Eastern Iran

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ABSTRACT

Background: This research aimed to estimate the prevalence of extended-spectrum b-lactamase-producing Enterobacteriaceae (ESBL-PE) in stool samples of patients with different types of cancer.

Materials and Methods: Stool samples or deep rectal swabs were collected from cancer cases from January 2017 to December 2018. After species identification, in order to detect ESBL-PE, double-disk synergy test (DD test) was used. Disk diffusion procedure was conducted to determine the susceptibility of bacteria to antimicrobials. Lastly, antibiotic resistance genes including *bla* genes were characterized via polymerase chain reaction (PCR).

Findings: Among 100 patients enrolled in this study, 50 (50%) were ESBL carriers. *Escherichia coli* was the most prevalent bacterium isolated (85%). Genotyping of ESBL-PE encoding genes using PCR showed that the prevalence rates of *bla*_{CTX-M} and *bla*_{CTX-M-15} genes were 94 (47 of 50) and 90% (45 of 50), respectively, which were higher than those of *bla*_{TEM} (80%, 40 of 50) and *bla*_{SHV} genes (34%, 17 of 50). There was no significant association between ESBL-PE fecal carriage and age ($p = .68$), sex ($p = .49$), time of diagnosis ($p = .21$), antibiotic therapy for the past three months ($p = .77$), and history of chemotherapy ($p = .49$). Finally, it was determined that cancer type was an associated risk factor for ESBL-PE fecal carriage in cancer patients.

Conclusion: This research emphasizes regular bacterial monitoring, and that antibiotic stewardship plans ought to be performed among cancer patients to prohibit further spread of ESBL-PE with confined therapeutic options.

Keywords: Enterobacteriaceae extended-spectrum beta-lactamase, Fecal carriage, Cancer patients, Iran.

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Introduction

Many studies have revealed that there is a strong relationship between malignancy and microbial infections [1]. Studies have shown that bacterial infections are the most current problems in cancer cases because they are likely to get immunodeficiency disorders owing to undernutrition, surgical or invasive procedures, chemotherapy, radiation therapy, and treatment interference [2]. Infectious diseases are one of the salient non-cancerous causes of death in cancer cases. In developing countries, infections cause the death of one in five cancer patients [1].

Infections caused by resistant Gram-negative bacteria are a major universal health challenge because of their difficult treatment and high mortality. The extended-spectrum beta-lactamase producing Enterobacteriaceae bacteria (ESBL-PE) have become a rather outstanding problem [1]. ESBLs display resistance to many antibiotics, such as cephalosporins, penicillins, and aztreonam [3]. Drug resistance in ESBL-PE isolates is related to the manufacture of resistance enzymes like beta-lactamases and extended-spectrum beta-lactamases, of which the CTX-M, TEM, and SHV beta-lactamases are the typical forms [4].

In cancer cases, the gastrointestinal tract is a main endogenous source of infectious organisms, and intestinal colonization is a prelude to intestinal transposition and systemic dispensation of bacteria [5]. In a study, 54% of children with various types of cancer in the oncology ward were carriers of ESBL, of which CTX-M of ESBL-type was the most common [6]. In a study in Spain, ESBL-producing *Escherichia coli* (ESBL-EC) fecal carriage was found in 29% of neutropenic patients with hematological malignancies, and previous antibiotic therapy in neutropenic cancer patients was determined to be effective in EC fecal colonization. Analysis of ESBL-

EC strains isolated from fecal samples indicated a polyclonal distribution with CTX-M superiority (CTX-M 82%, SHV 15%, TEM 3%) [7, 8]. In another study in Spain, ESBL-EC bacteremia was common among cancer patients, particularly those under antibiotic pressure, and most ESBL-EC strains contained the CTX-M group enzymes [9]. In a study conducted during 2012-2018 in Korea, ESBL-PE accounted for 12.8% of sepsis cases among patients suffering from chemotherapy-induced neutropenia [10]. A study in western China showed that ESBL-PE members were mostly (72.8%) MDR strains isolated from cancer patients with nosocomial infections [2]. In 2014, ESBL-PE isolates were shown in a study to be the most prevalent bacteria causing bloodstream infections in hematological cancer cases [11]. In another study conducted in Spain, ESBL-EC fecal carriage was regarded as a risk factor for bloodstream infections (BSI) in immunosuppressed patients with higher mortality compared with non-ESBL-EC [6]. ESBL-PE fecal carriage is an important risk factor for subsequent infections by these microorganisms [6]. Infections caused by these resistant organisms have limited treatment options, have a poorer prognosis, and are less cost-effective. Hence, inability to detect these resistant enzymes contributes to their uncontrolled dispersion, outbreaks, and sometimes avoidable remedial failures [12]. As far as we know, no research has been conducted in Iran to assess the prevalence of ESBL-PE fecal carriage and its relationship with infections in cancer patients.

Objectives: the objective of this research was to study the prevalence of gastrointestinal colonization with ESBL-PE in patients suffering from various types of cancer and to ascertain its relationship with age, sex, type of malignancy, duration of cancer, and history of antibiotic consumption in these patients.

Materials and Methods

Sampling: In this cross-sectional study, fecal samples were collected from 100 cancer patients (breast, gastrointestinal, genitourinary, skin, and lung cancers) who referred to the oncology units of two educational hospitals (Imam Reza and Vali-e-Asr). The average age of cancer cases was 57.11±16.14 (SD) years, of whom 64% were female, and the rest were male. One hundred stool samples or deep-rectal swabs (disable patients) were collected from January 2017 to December 2018 and promptly transported to the laboratory in a thermostatic container. All samples were stored at 4 °C. Statistical information was collected from all patients, including age, sex, and history of antibiotic use within the past three months, time of diagnosis, history of anticancer chemotherapy, and cancer type.

The inclusion criteria were as follows: a) patients who accepted to take part in this study, and b) all patients who had pathology-confirmed cancer.

Exclusion criteria were also as follows: a) fecal samples of patients who died before the end of the study, b) patient dissatisfaction to complete the study, and c) intake of probiotics within four weeks prior to the study, which might have influenced the gut milieu.

Microbial Identification: Stool samples were vortexed in 1 mL of sterile phosphate-buffered saline (PBS). Then the samples were cultured on two plates, containing MacConkey agar supplemented with cefotaxime (4 µg/mL) and ceftazidime (4 µg/mL), and incubated at 37 °C for 24 hours [6]. Bacterial colonies were identified based on standard biochemical tests, including oxidase, triple sugar iron agar (TSI), Simmon citrate, indole production, urease, and methyl red/Voges-Proskauer (MR-VP). All media were provided from Merck Co., Darmstadt, Germany. Then colonies with morphologies similar to Enterobacteriaceae were isolated and stored in tryptic soy broth

(TSB) with 15% glycerol at -25 °C.

Antimicrobial Sensitivity Testing: Antimicrobial sensitivity testing was conducted for Enterobacteriaceae isolates by applying the disc diffusion method under the guidelines of the Clinical and Laboratory Standards Institute (CLSI, 2017) using commercially accessible discs (Mast, UK), including cefotaxime (30 µg), ceftazidime (30 µg), ciprofloxacin (5 µg), gentamicin (10 µg), imipenem (10 µg), amikacin (30 mg), and levofloxacin (5 µg). *E. coli* ATCC 25922 was used as a quality control strain [13].

Phenotypic Detection of ESBLs: A double-disk synergy test (DD test) was applied to screen for ESBL-producing isolates, such as ceftazidime and cefotaxime alone, ceftazidime + clavulanic acid, and cefotaxime + clavulanic acid (Mast group, UK) [14]. Bacterial isolates showing positive results in the DD test were further investigated using PCR to screen for ESBL determinants as described below. *E. coli* ATCC 25922 and *Klebsiella pneumonia* ATCC700603 were used as the ESBL-negative and ESBL-positive controls, respectively.

Polymerase Chain Reaction (PCR) for Detection of ESBL Genes

DNA Extraction: Total DNA was extracted from fresh colonies of Enterobacteriaceae isolates by boiling approach as described previously [15]. Briefly, 2 or 3 colonies of an overnight culture of each isolate on nutrient agar (Merck Co., Darmstadt, Germany) were suspended in 500 µL of DNase- and RNase-free water. The suspension was boiled at 100 °C for 10 min in a dry block incubator and then centrifuged at 14,000 g for 10 min. Subsequently, 0.5 mL of the supernatant was used as a DNA template for PCR. The extracted DNA was stored at 20- °C until used for analysis [16].

PCR Protocol: The existence of *bla*_{CTX-M}, *bla*_{TEM}, *bla*_{SHV}, and *bla*_{CTX-M-15} in phenotypic ESBL-positive isolates in DDT was examined

by PCR using specific primers delineated in Table 1. The PCR amplification was carried out within a PeqSTAR 2X thermal cycler (PEQLAB, Germany) in a final volume of 20 μ L containing 10 μ L of Taq 2X Master Mix, 1 μ L of every primer (10 pmol), 2 μ L of sample DNA, and 6 μ L of nuclease-free water. The PCR thermal cycling conditions were as follows: an initial denaturation step at 94 °C for 5 min, followed by 35 cycles of denaturation at 95 °C for 35 s, annealing at different temperatures (Table 1) for 35 s, extension at 72 °C for 30 s, and a terminal extension step at 72 °C for 5 min. The amplicons were analyzed using agarose gel electrophoresis, stained with green viewer stain (parstous Co., Tehran, Iran), and visualized using an ultraviolet gel documentation device (Herolab, Germany). A positive control factor was provided from Pasteur Institute of Iran.

Statistical Analysis: SPSS software Version 22.0 was utilized for statistical analysis. The Chi-square test and Fisher's exact test, where appropriate, were conducted to compare variables. A p -value < .05 was considered statistically significant.

Findings

Characteristics of the studied population:

During this research, 100 patients with

various types of cancer, who referred to the oncology units of two hospitals in Birjand city, eastern Iran, were screened for fecal ESBL-PE carriage. The female to male ratio was 1.7 (64 to 36), and the average age of patients was 57.11 \pm 16.14 (SD) years. The most common cancers among patients were gastrointestinal, breast, genitourinary, skin, and lung cancers with prevalence rates of 40 (%40), 35 (%35), 15 (%15), %33), and 3 (%3) cases, respectively. Only %35 of patients had received antibiotics in the past three months prior to sampling. Most subjects (%80) had a history of cancer chemotherapy. In %51 of patients, the time of cancer diagnosis was related to the past two to five years. The clinical and demographic profiles of the subjects are exhibited in Table 2.

Species Identification: During the study period, in culture medium containing cefotaxime and ceftazidime, bacterial growth was observed in 54 (54%) out of 100 samples tested, and a total of seven different colonies were isolated. Based on colony morphology and conventional biochemical tests, *E. coli* (46, 85 %) was the most prevalent bacterium isolated. The prevalence rate of other bacteria was as follows: 1(1.8%) *K. oxytoca*, 3(5.5%) *Citrobacter freundii*, 1(1.8%) *Citrobacter amaloticus*, 1(1.8%) *Morganella morganii*,

Table 1) Target *bla* genes and used primers in this research

Primer	Sequence (5'-3')	Products Sizes (bp)	Annealing (°C)	Reference
<i>bla</i> _{TEM}	Fw-ATGAGTATTCAACATTTCCG Rv- CTGACAGTTACCAATGCT TA	868	54	(23)
<i>bla</i> _{SHV}	Fw- ATGCGTTATATTCGCCTGTGTAT Rv- TTAGCGTTGCCAGTGCTCGATCAG	868	56	(24)
<i>bla</i> _{CTX-M}	Fw- ATGTGCAGCACCAGTAAAGT Rv- ACCGCGATATCGTTGGTGG	542	55	(25)
<i>bla</i> _{CTX-M-15}	Fw- ATAAAACCGGCAGCGGTG Rv- GAATTTTGACGATCGGGG	483	55	(26)

Table 2) The baseline characteristics and clinical features of the study population

Data		Frequency (f)	Percentage (%)
Sex	Male	36	36
	Female	64	64
Age (in years)	≤ 40	12	12
	41-59	48	48
	60 ≤	40	40
Cancer types	Breast	35	35
	Gastrointestinal	40	40
	Genitourinary	15	15
	Skin	3	3
	Lung	3	3
	Others	4	4
Time of diagnosis (in years)	< 1	35	35
	1	7	7
	2-5	51	51
	5 <	7	7
Prior antibiotic use (past three months)	Positive	38	38
	Negative	62	62
History of cancer chemotherapy	Positive	80	80
	Negative	20	20

and 2(3.7%) *Pseudomonas aeruginosa*. **Antimicrobial susceptibility testing and Phenotypic Detection of ESBLs:** Antibiotic susceptibility and DDT tests were performed on 54 Enterobacteriaceae isolates. Antimicrobial sensitivity testing showed that among the seven antimicrobial agents tested, resistance rates to β -lactam antibiotics including cefotaxime, ceftazidime, and imipenem were 51 (94.4%), 32 (59.3%), and 1 (1.9%) cases, respectively. Also, levels of resistance to non- β -lactam antibiotics including gentamicin, ciprofloxacin, levofloxacin, and amikacin were 8 (14.8%), 19 (35.2%), 19 (35.2%),

and 2 (3.7%) cases, respectively. The highest resistance of isolates was observed against cefotaxime, and the lowest resistance was against imipenem (Figure 1).

The results of DDT showed that 50 (92.5%) isolates were ESBL positive, whereas the remaining 4 (7.4%) isolates were not ESBL producer.

In addition, there was no considerable correlation between ESBL-PE fecal carriage and age (p -value= .68), sex (p -value= .49), time of diagnosis (p -value= .21), antibiotic therapy in the past three months (p -value= .77), and chemotherapy history (p -value= .49) in this research (Table 3).

Table 3) Comparative characteristics of the study population according to the extended-spectrum b-lactamase-producing Enterobacteriaceae fecal carriage

Demographic Variable	ESBL-Producing Enterobacteriaceae % (n)	Non ESBL-Producing Enterobacteriaceae % (n)	P-value	F-Value
Gender				
Male	58.3 (21)	17.58 (15)	.49	1.58
Female	45.3 (29)	57.4 (35)		
Age group (years)				
≤ 40	41.7 (5)	58.3 (7)	.68	2.28
41-50	52.1 (25)	9.65 (23)		
60 ≤	50.0 (20)	11.12 (20)		
Prior antibiotic use (past three months)				
Prior antibiotic use	47.7 (18)	56.2 (20)	.77	0.57
No prior antibiotic use	51.6 (32)	13.83 (30)		
Prior chemotherapy	52.5 (42)	16.73 (38)	.49	1.33
No Prior chemotherapy	40.0 (8)	60.0 (12)		
Time of diagnosis (in years)				
< 1	42.9 (15)	57.1 (20)	.21	7.77
1-5	25.14 (33)	35.18 (25)		
5 <	28.6 (2)	71.4 (5)		

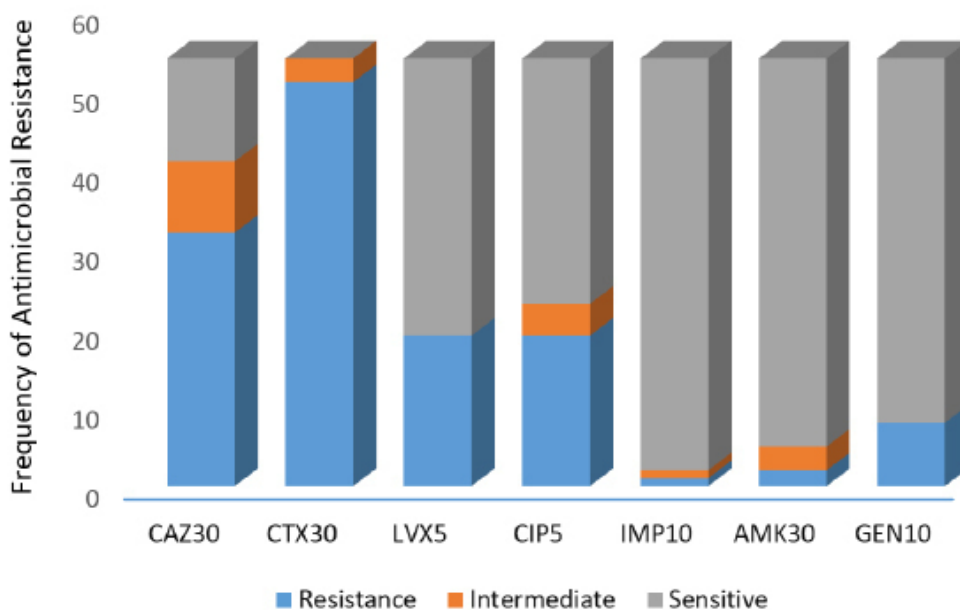


Fig. 1. Frequency of antimicrobial resistance of isolates from patients with cancer. S: susceptible, I: intermediate, R: resistant, GM₁₀: gentamicin, AMK₃₀: amikacin, IMP₁₀: imipenem, CIP₅: ciprofloxacin, LVX₅: levofloxacin, CTX₃₀: cefotaxime, CAZ₃₀: ceftazidime

Prevalence of β-Lactamase-Producing Isolates: PCR amplification was used to verify ESBL production in 50 DDT-positive strains. The presence of ESBL-encoding

genes in these 50 isolates was verified through molecular analysis. Of 100 patients with various types of cancer participated in this study, 50% were ESBL-PE carrier.

The existence of *bla*_{TEM}, *bla*_{SHV}, *bla*_{CTX-M}, and *bla*_{CTX-M-15} genes in each organism was checked by PCR as explained before. The ESBL genes identified were as follows: *bla*_{CTX-M} (47 of 50; 94%), *bla*_{CTX-M-15} (45 of 50; 90%), *bla*_{SHV} (17 of 50; 34%), and *bla*_{TEM} (40 of 50; 80%) (Figure 2). The CTX-M group ESBL enzymes were identified in most of the isolates.

Gastrointestinal and breast cancers were the foremost diagnosed cancers among patients. It was also determined that cancer type had the most contribution to the fecal carriage of ESBL-PE. A remarkable rise in the prevalence

of ESBL-PE fecal carriage was found in gastrointestinal and genitourinary cancers. In other words, the prevalence rate of ESBL-PE fecal carriage in cancer cases was 50%, of which 40% was observed in patients with breast cancer, 67.5% in gastrointestinal cancer, 53.3% in genitourinary cancer, and 25% in other cancers (skin and lung) (Figure 3).

Discussion

Colonization of the gastrointestinal tract (GIT) with ESBL-PE is a significant risk factor for subsequent infections caused by

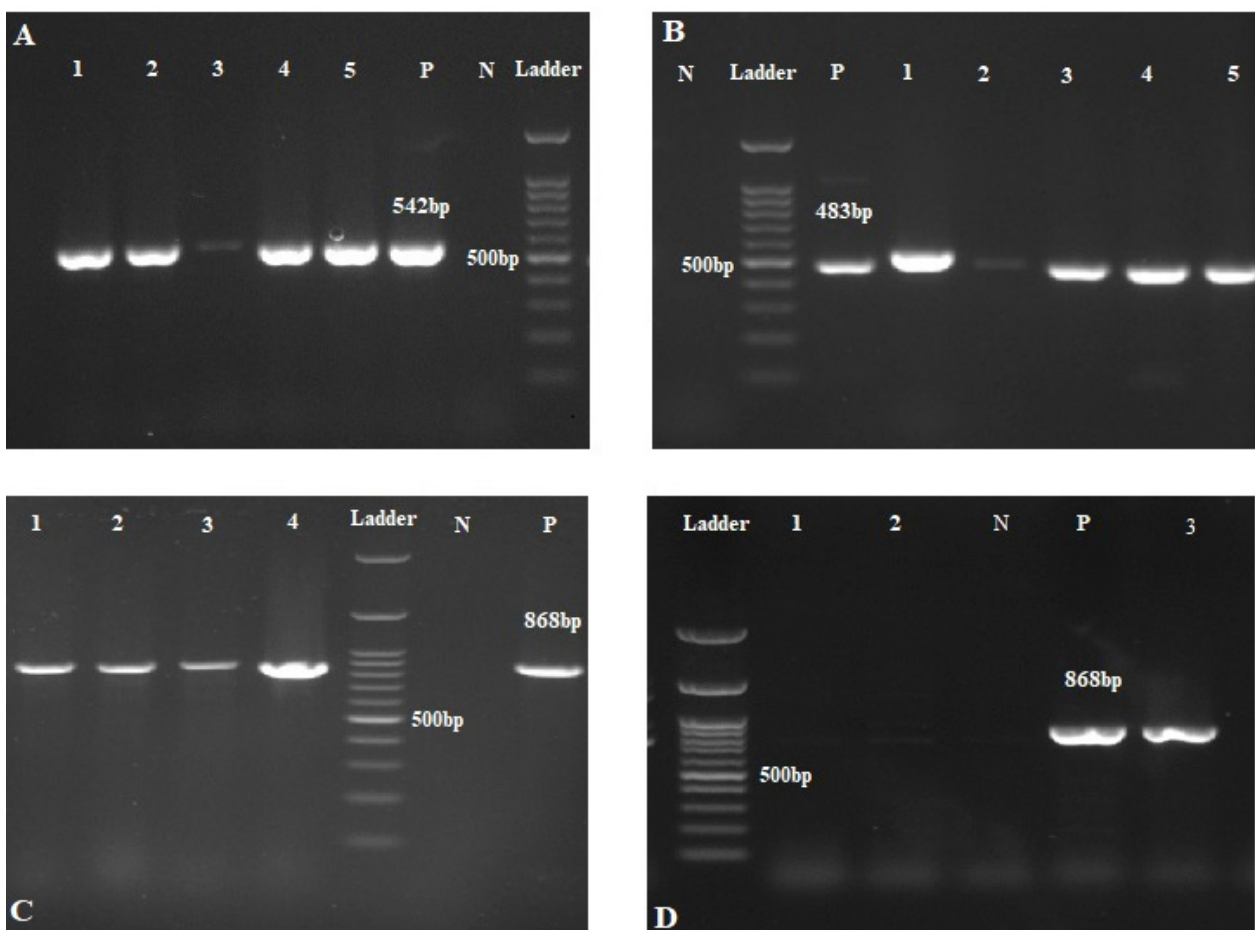


Fig. 2) PCR amplification products of extended-spectrum β -lactamase genes from Enterobacteriaceae isolates. (A) PCR amplification of *bla*_{CTX-M} gene. Lane 1, 2, 3, 4, and 5: clinical isolates; Lane P: positive control; Lane N: negative control; and Lane ladder: DNA marker (100 bp). (B) PCR amplification of *bla*_{CTX-M-15} gene. Lane 1, 2, 3, 4, and 5: clinical isolates, Lane P: positive control; Lane N: negative control; and Lane ladder: DNA marker (100 bp). (C) PCR amplification of *bla*_{TEM} gene. Lane 1, 2, 3, and 4: clinical isolates; Lane N: negative control; Lane P: positive control; and Lane ladder: DNA marker (100 bp). (D) PCR amplification of *bla*_{SHV} gene. Lane 1, 2, and 3: clinical isolates; Lane N: negative control; Lane P: positive control; and Lane ladder: DNA marker (100 bp)

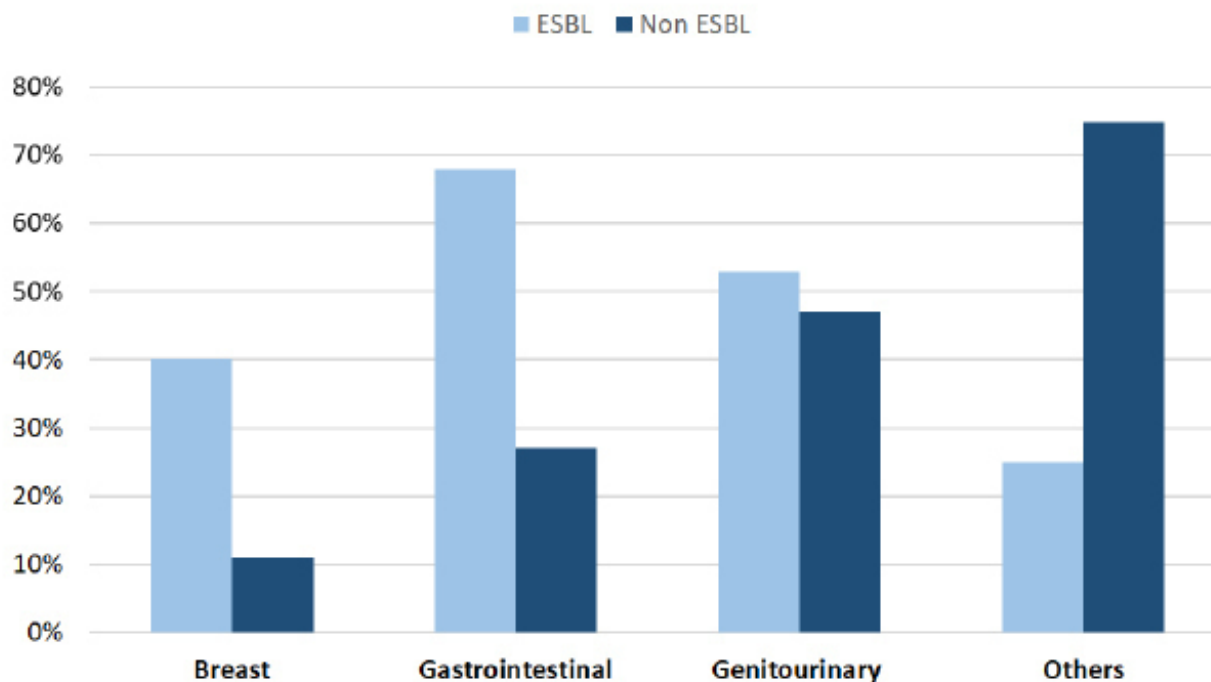


Fig. 3) The prevalence of ESBLs fecal carriage in different cancers

these resistant pathogens. GIT colonization is worrying in cancer cases because cytotoxic chemotherapy and prolonged use of broad-spectrum antibiotics alter the gut microbiome. The prevalence of ESBL-PE varies globally and is rapidly changing over time [6]. In eastern Iran, no data are available about ESBL fecal carriage in patients with cancer; thus, this research is the first study displaying alarmingly high prevalence of ESBL-PE colonization.

The prevalence of ESBL-PE fecal carriage in this study was 50%, which is close to the finding reported by Medboua-Benbalagh et al. (2017) in an oncology pediatric unit in Algiers (54%) [6], but higher than that reported (31.8%) in neutropenic patients with cancer in Spain [17]. It is also higher than the result previously recorded by Golzarri et al. (2019) (21%) in patients with gastrointestinal and gynecological malignancies in Mexico City [18]. This vast variation in the prevalence rate of ESBL-PE fecal carriage is possibly related to the diversity in the type of samples analyzed.

Overall, ESBL-producing *E. coli* strains were the most prevalent bacteria isolated from the fecal samples of 100 cancer patients studied. This finding is consistent with the results of recent studies in Spain, Mexico, Algeria, and Lebanon, which have reported alarmingly high levels of fecal colonization by ESBL-producing *E. coli* strains among neutropenic cases [6, 17-19].

The antibiotic susceptibility testing results in previous research by Montazeri et al. (2020) manifested high resistance of ESBL-PE strains, causing bloodstream infections (BSIs) in cancer cases, to b-lactam antibiotics like ceftazidime (85.7%) and cefotaxime (76.1%). In their study, amikacin, gentamicin, fluoroquinolones (ciprofloxacin, levofloxacin), and imipenem were identified as suitable antibiotics for the treatment of Enterobacteriaceae infections in cancer cases in the southwest of Iran due to their low resistance rate [16]. These findings are consistent with the present study results. However, these outcomes contradict the results of another research carried out by

Medboua-Benbalagh [6]. Different patterns of antibiotic usage in various geographical areas and the difference in the age of the studied populations could be the major reasons for the discrepancy between these two studies results.

In the present research, *bla*_{CTX-M} with a prevalence rate of 94% was the most common gene among the isolates, followed by *bla*_{CTX-M-15} and *bla*_{TEM} gene with a prevalence rate of 90 and 80%, respectively, this finding is in line with the findings of other studies displaying the significance of *bla*_{CTX-M} in different nations [16-18, 20].

In this study, the risk factors associated with ESBL-PE fecal carriage in cancer cases were also investigated. In line with several studies results [6, 16], there was no significant relationship between ESBL-PE fecal carriage and sex, age, history of chemotherapy, and time of diagnosis. No significant relationship was also found between ESBL-PE fecal carriage and previous treatment with antimicrobial drugs. These findings are in line with those previously published by Knudsen et al. [21] but in contrast with those reported by other researchers [6, 9, 17, 18, 20]. The discrepancy between different studies results could be due to differences in the study population, the amount of antibiotics used, and the type of antibiotics used in different countries, suggesting that the risk factors of bacterial resistance are multifactorial and complex in nature.

According to previous research, ESBL-PE colonization is associated with the development of bloodstream infections and higher mortality in patients with weakened immune systems [6, 16, 22]. Thus, investigation of colonization with resistant bacteria in a variety of cancers might help specify infection-control practices and prevent further spread of ESBL-PE with limited therapeutic options. In this study, the prevalence of ESBLs varied dramatically

between different cancers, ranging from 25% in skin and lung cancers to 67.5% in gastrointestinal cancer. Cancer type and ESBL-PE fecal carriage were found to have a considerable positive relationship. The higher rate of fecal colonization observed in patients with gastrointestinal cancer could be linked to regular use of endoscopic diagnostic techniques and management of gut obstruction. Contaminated endoscopes as medical apparatuses are often related to increased colonization rates with antibiotic-resistant bacteria. To the best of our knowledge, a limited number of studies have evaluated the association between the cancer type and ESBL-PE fecal carriage in cancer patients. In Medboua-Benbalagh's research, the rate of ESBL-PE fecal carriage in children with hematological malignancies was higher than in children with solid tumors, which confirms this study findings [6]. Montazeri et al. (2020) investigated the effect of cancer type on the prevalence of ESBL-PE causing bloodstream infections in cancer patients in southwestern Iran, and there was no relationship between the two [16].

There are some restrictions that must be mentioned. First, there are numerous potential problems when screening fecal samples. Although stool samples naturally produce a high yield of organisms, they are not always available. Second, the fecal samples studied in this research were gathered some years ago, and the restriction of the current research is the absence of information from the present time. It would be interesting to conduct such research right now in Iran.

Conclusion

In summary, the incidence of ESBL-PE fecal carriage in patients with various types of cancer was 50%. The present study findings demonstrated that most ESBL-PE strains

contained the CTX-M group enzymes. The cancer type had the most contribution to the fecal carriage. This research provides new information on the hidden reservoirs and features of the study population, which could aid in the development of infection control policies. Thus, to avoid the further distribution of ESBL-PE that has restricted therapeutic options, cancer patients should be controlled, and antibiotic stewardship plans ought to be performed.

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Conflicts of Interests: All authors state that they have no competing interest.

Authors Contribution: Conceptualization: MHN; data curation: MHN, ARS, and PA; formal analysis: FH and PA; funding acquisition: MHN; investigation: PA and MM; methodology: MHN, ARS, and PA; project administration: MHN; resources: MHN; software: PA and FH; supervision: MHN and ARS; writing of the original draft: PA; writing-review and editing: MHN.

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