Research Article

Genetic Virulence Determinants and Antimicrobial Susceptibility Profile of *Escherichia coli* Isolated from Some Milk Products

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Abstract | *Escherichia* (*E.*) *coli* is a highly versatile bacterial species habitats intestinal tract of warm-blooded animals as a normal flora and it can cause severe illnesses in different animal species and human being. Dairy products are considered a source of *E. coli* to humans. In humans, it could cause variety of diseases ranges from bloody diarrhea to hemolytic uremic syndrome. This study aimed at studying the prevalence of *E. coli* in yoghurt, kariesh cheese and cream, inspecting the prevalent *E. coli* serogroups, investigating their antimicrobial susceptibility profile using the disk diffusion test and determining some of its virulence genes. A total of 155 samples were collected (50, 50 and 55 from yoghurt, cream and kariesh cheese, respectively) from local markets in El-Fayoum Governorate, Egypt. The prevalence of *E. coli* in yoghurt, cream and kariesh cheese were 12.0, 56.0 and 61.8%, respectively. There were 11 different serogroups of *E. coli* amongst the inspected isolates. Serogroups O: 55, O: 114 and O: 125 were identified in the whole examined products, while serogroups O: 26, O: 27 and O: 78 were identified in yoghurt and kariesh cheese only. Antimicrobial resistance against ampicillin, streptomycin, trimethoprim-sulfamethoxazole, cefotaxime, nalidixic acid, tetracycline, and amoxicillin-clavulanic acid were 11.8, 10.3, 8.8, 7.4, 5.9, 4.4, and 4.4%, respectively. Moreover, multidrug resistance was noted in 10.3% of the inspected *E. coli* isolates. PCR revealed the presence of *ast*A, *eae*A, *stx*1 and *stx*2 genes in 100, 50, 20 and 10%, respectively of the tested isolates. The present study clarify that yoghurt, kariesh cheese and cream to be potential sources of various *E. coli* pathotypes harboring virulence factors able to induce lethal diseases in humans. Moreover, multidrug resistant strains of *E. coli* that even if non-pathogenic will participate in establishing resistance in gastrointestinal tract bacterial community and subsequently environment. So, there is a fundamental need to follow the implementation of both good hygiene and manufacturing practices as well as application of strict hazards analysis and critical control point in dairy products industry for the sake of human safety.

Keywords | *E. coli*, Virulence, Genes, Serogroupe, Cheese

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INTRODUCTION

Escherichia coli (*E. coli*) is Gram-negative bacilli belongs to the family Enterobacteriaceae. *E. coli* strains habitat intestinal tract (Lara et al., 2016) of both animal and humans normally as a non-pathogenic bacilli. Although most of *E. coli* strains are non-pathogenic, some strains are well armed with a variety of virulence factors that are

diverge in accordance to the pathotype of *E. coli*.

Pathogenic *E. coli* have been classified into two categories; the diarrheagenic *E. coli* (DEC) and the extraintestinal pathogenic *E. coli* (ExPEC). Among the diarrheagenic *E. coli*, there are currently six categories including enteropathogenic *E. coli* (EPEC), enterotoxigenic *E. coli* (ETEC), enteroinvasive *E. coli* (EIEC), enteroaggregative *E. coli* (EAEC), diffusively adherent *E. coli* (DAEC) and enterohemorrhagic *E. coli* (EHEC)/Shiga toxin-producing *E. coli* (STEC) (Xiaodong, 2010). While extra-intestinal pathogenic *E. coli* (ExPEC) can be classified into three categories, namely, uropathogenic *E. coli* (UPEC) causing urinary tract infection (UTI), meningitis-associated *E. coli* (MNEC) and necrotoxigenic *E. coli* (NTEC) which produces cytotoxic necrotizing factor (CNF) (Kaper et al., 2004).

Milk and milk products including (yoghurt, kariesh cheese and cream) are consumed worldwide. They classified as sources of great biological value protein, principal vitamins and minerals (Pereira, 2014). Consumption of raw milk and raw-milk products are widely distributed in several countries as well as Egypt (Ayad et al., 2004). On the other hand, they are considered as source of possibly injurious bacteria to humans, such as pathogenic *E. coli* (Oliver et al., 2005). *E. coli* can gain access to milk via fecal contamination or via direct secretion (mastitis) from udder into milk (Stephan and Kühn, 1999).

STEC represent a dangerous public health problem worldwide initiating several human gastrointestinal tract diseases, including watery or bloody diarrhea, and may lead to a life-threatening disease, such as haemorrhagic colitis (HC), thrombotic thrombocytopenic purpura (TTP) and hemolytic uremic syndrome (HUS) (Kalid and Andreoli, 2018). STEC strains yield two powerful cytotoxins initiating tissue damage in humans called Shiga toxins or verotoxins (*stx*1/*vt*1 and *stx*2/*vt*2) (Li et al., 2017). *Stx*2 producing strains are frequently linked to more severe infections (Muniesa et al., 2004).

Other virulence factor likewise, outer membrane protein intimin that is encoded by the *eae*A gene and firmly attach and form attaching and effacing lesions to intestinal epithelial cells (Awad et al., 2020). Another dangerous aspect of pathogenic *E. coli* is the presence of the *ast*A gene encoding enteroaggregative heat-stable enterotoxin 1 (EAST1) which was primarily distinguished in EAEC (Dubreuil, 2017). Afterward, *ast*A gene was detected in another DEC pathotypes including EPEC, ETEC, and EHEC (Ménard and Dubreuil, 2002). The *ast*A gene possibly is a significant virulence factor in DEC which could be injurious to humans (Hinenoya et al., 2014).

The present study aimed at investigating some dairy products (yoghurt, kariesh cheese and cream) manufactured and retailed under market situations for the prevalence of *E. coli*, prevalent serogroups, presence of some virulence genes. Additionally, to study their antimicrobial susceptibility profile against antimicrobial agents of veterinary and humans medicine concern.

MATERIALS AND METHODS

Samples

A total of 155 samples from different milk byproducts (50 yoghurt, 55 kariesh cheese and 50 cream samples) were collected from different supermarkets, retail and dairy shops in Al-Fayoum governorate, Egypt during the period from August 2019 to February 2020. Samples were transferred in sterile containers to the laboratory and analyzed directly on arrival for the isolation of *E. coli*.

Isolation and identification of *E. coli*

Isolation and biochemical identification of *E. coli* was done according to Collee et al. (1996).

Detection of hemolytic activity of the identified *E. coli* isolates

The ability of *E. coli* isolates to produce different types of hemolysin was phenotypically investigated using sheep blood agar 7% (Collee et al., 1996).

Serogrouping of the isolated *E. coli*

Serogrouping of *E. coli* isolates recovered from different milk byproducts was performed in accordance to Ewing (1986). I was performed in Department of Serology, Animal Health Research Institute, Agricultural Research Center, Egypt.

In vitro antimicrobial susceptibility testing of the identified *E. coli* strains

The isolated *E. coli* strains were investigated for their antimicrobial susceptibility profile using disk diffusion test against different antimicrobial classes of veterinary and human being significance. Second generation cephalosporin (cefoxitin, 30µg); third generation cephalosporin (cefotaxime, 30 µg); penicillin-inhibitor combination (amoxicillin-clavulanic acid, 30 µg); aminoglycosides (gentamicin, 10 µg and streptomycin, 10 µg); tetracyclines (tetracycline 10 µg); quinolone (nalidixic acid 30 µg); fluoroquinolone (ciprofloxacin, 5 µg); carbapenem (imipenem, 10 µg); and folate pathway antagonist (trimethoprim-sulfamethoxazole, 25 µg). All antimicrobial disks were obtained from Oxoid, UK. The *in vitro* antimicrobial susceptibility profiling and results interpretation were performed according to CLSI (2019).

Table 1: Oligonucleotide primer sequences of target genes specific for *E. coli*.

Gene	Primer sequence (5'-3')	Amplicon size	Reference
stx1	ACACTGGATGATCTCAGTGG	614 bp	Dipineto et al. (2006)
	CTGAATCCCCCTCCATTATG		
stx2	CCATGACAACGGACAGCAGTT	779 bp	
	CCTGTCAACTGAGCAGCACTTTG		
eaeA	ATGCTTAGTGCTGGTTTAGG	248 bp	Bisi-Johnson et al. (2011)
	GCCTTCATCATTTCGCTTTC		
astA	CCATCAACACAGTATATCCGA	110 bp	Piva et al. (2003)
	GGTCGCGAGTGACGGCTTTGT		

Table 2: PCR cycling conditions of the different primer sets.

Gene	Primary denaturation	Secondary denaturation	Annealing	Extension	No. of cycles	Final extension
stx1 and stx2	94°C 5 min.	94°C 30 sec.	58°C 40 sec.	72°C 45 sec.	35	72°C 10 min.
eaeA	94°C 5 min.	94°C 30 sec.	51°C 30 sec.	72°C 30 sec.	35	72°C 7 min.
astA	94°C 5 min.	94°C 30 sec.	55°C 30 sec.	72°C 30 sec.	35	72°C 7 min.

Detection of some virulence genes in the prevalent *E. coli* serogroups isolated from dairy products using polymerase chain reaction (PCR)

Presence of *ast*A, *eae*A, *stx*1, and *stx*2 in the most prevalent serogroups of the isolated *E. coli* was done using PCR .

Preparation of DNA template

DNA template was obtained from overnight pure culture using QIAamp DNA Mini Kit (Catalogue no.51304) from Qiagen.

Amplification procedure

PCR reactions were performed in volumes of 25µL. Primers were obtained from Metabion (Germany) and master mix from Takara (Catalogue no. RR310). Table 1 reveals the used primer pairs for each gene, amplicon size and references used and Table 2 shows the cycling condition for each primer pairs. Ten microliters of the reaction products were analyzed by electrophoresis on 1% agarose gel containing ethidium bromide and results were visualized in a gel documentation system.

Statistical analysis

ANOVA test was used to investigate the prevalence of *E. coli* in different dairy products. Statistical significance was considered if $p \leq 0.05$. All statistical comparisons were performed using IBM SPSS® Statistics software version 22.

RESULTS AND DISCUSSION

Prevalence of *E. coli* in different dairy products

E. coli prevalence (Table 3) differed according to the niche of dairy products. The highest prevalence (61.8%) was noted in kariesh cheese followed cream (56%), while the least prevalence was reported amongst yoghurt samples (12%). The overall prevalence of *E. coli* in the examined samples of the dairy products was 43.9%.

Table 3: Prevalence of *E. coli* isolated from dairy products.

Dairy product	Samples No.	*E. coli*	
		No.	%*
Yoghurt	50	6	12.0§
Kariesh cheese	55	34	61.8
Cream	50	28	56.0
Total number	155	68	43.9

*%: Percentage was calculated according to the corresponding number of examined samples; §: Prevalence of *E. coli* in yoghurt was statistically lower than those reported in kariesh cheese and cream ($p<0.05$).

Prevalence of *E. coli* in yoghurt was statistically lower than those reported in kariesh cheese and cream ($p<0.05$). In contrast, kariesh cheese and cream *E. coli* prevalence difference was not statistically different (p>0.05).

Hemolytic activity of *E. coli* isolates

Alpha hemolysis was the only type of hemolysis phenotypically detected in *E. coli* isolated in the present study. Alpha-hemolytic activity was reported in 16 (23.5%) out of the inspected 68 isolates (one isolate recovered from cream and 15 isolates from kariesh cheese), while the remaining isolates were non-hemolytic i.e. gamma-hemolytic.

Serogrouping of *E. coli* isolates

Table 4 reveals the presence of 11 serogroups of *E. coli* amongst investigated 20 isolates that were selected to

represent the examined dairy products (yoghurt, 5; kariesh cheese, 7 and cream, 8) under study (Table 4). Serogroups O55, O114 and 125 were reported in the investigated three dairy products.

Table 4: Serogrouping of *E. coli* isolates recovered from dairy products.

Product	No. of isolates	Serogroup
Yoghurt	5	O25
		O55
		O114
		O125
		O128
Kariesh cheese	7	O26
		O27
		O55
		O78
		O114
		O124
		O125
Cream	8	O26
		O27
		O55
		O78
		O86
		O114
		O125
		O148

Antimicrobial susceptibility testing of *E. coli* recovered from dairy products

The *in vitro* antimicrobial susceptibility testing revealed diverse susceptibility/resistance behavior of the investigated *E. coli* against the tested antimicrobial agents (Table 5). All 68 tested isolates were 100% sensitive to gentamicin, imipenem, and cefoxitin. On the other hand, the resistance rates against ampicillin, streptomycin, trimethoprim-sulfamethoxazole, cefotaxime, nalidixic acid, tetracycline and amoxicillin-clavulanic acid were 11.8, 10.3, 8.8, 7.4, 5.9, 4.4 and 4.4%, respectively. Additionally, multidrug resistance was noted in seven out of 68 (10.3%) of the inspected *E. coli* isolates.

Detection of some virulence genes in the prevalence *E. coli* serogroups isolated from dairy products

The presence of *ast*A gene was confirmed in the isolates (100%) under test (Figure 1), while *eae*A was only defined in five out of ten (50%) investigated isolates (Figure 2). Regarding to shiga toxins, *stx*1 and *stx*2 were only defined in one (10%) and two (20%) of the tested isolates, respectively (Figure 3). Results clarify all isolates harboring shiga toxin genes also harbored *ast*A and *eae*A genes.

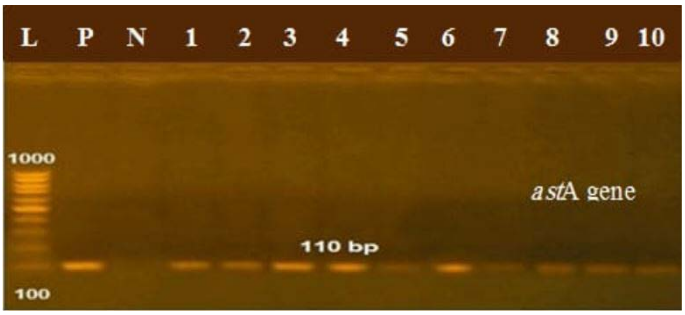

Figure 1: Lanes 1:10 positive amplification of *ast*A gene at 110 bp P: positive control; N: negative control; L: DNA ladder.

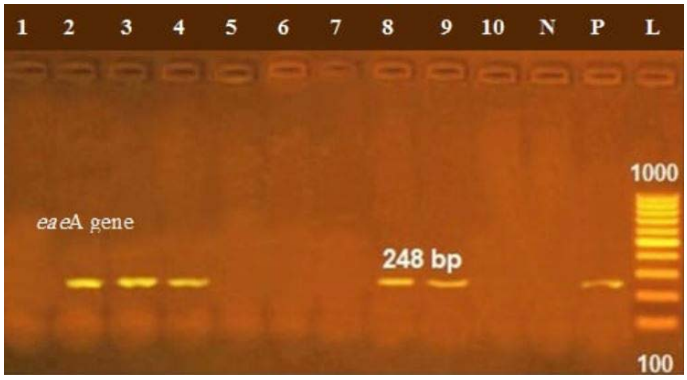

Figure 2: Lanes 2, 3, 7, 8, 9 positive amplification of *eae*A gene at 248 bp; P: positive control; N: negative control; L: DNA ladder.

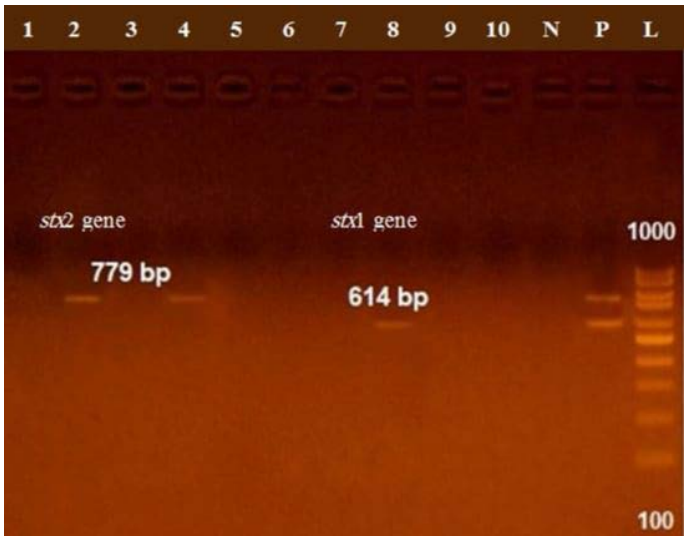

Figure 3: Lane 8 positive amplification of *stx*1 gene at 614 bp Lanes 2, 4 positive amplification of *stx*2 gene at 779 bp; P: positive control; N: negative control; L: DNA ladder.

Dairy products like yoghurt, cheese, and cream are widely consumed and markets have existed for them in many parts of the world for many generations. Dairy products over and above they are of high nutritional value for humans, they also offer an apt niche for bacterial growth likewise *E. coli*.

Table 5: Results of antimicrobial sensitivity test to 68 *E. coli* isolates from dairy products using the disk diffusion method.

Class	Antimicrobial agent	Sensitive		Intermediate		Resistant	
		No.	%	No.	%	No.	%
Penicillin	Ampicillin	60	88.2	0	0	8	11.8
ß-lactam/β-lactamase inhibitor combinations	Amoxicillin-clavulanic acid	65	95.6	0	0	3	4.4
Third generation cephalosporins	Cefotaxime	63	92.6	0	0	5	7.4
Second generation cephalosporins	Cefoxitin	68	100	0	0	0	0
Carbapenems	Imipenem	68	100	0	0	0	0
Aminoglycosides	Gentamicin	68	100	0	0	0	0
	Streptomycin	54	79.4	7	10.3	7	10.3
Tetracyclines	Tetracycline	63	92.6	2	2.9	3	4.4
Quinolones	Ciprofloxacin	66	97.1	2	2.9	0	0
	Nalidixic acid	64	94.1	0	0	4	5.9
Folate pathway antagonist	Trimethoprim-sulfamethoxazole	61	89.7	1	1.5	6	8.8

%: Percentages were calculated in relation to the total number of tested isolates.

E. coli finds its way to milk and dairy products either, endogenously from the udder of diseased animal and or exogenously via direct contact with infected herds, environment or personnel (Farzana, 2009). In another consequence, *E. coli* is one of the main indicator organisms used for evaluating the quality of food (Anderson et al., 2006).

The present study revealed a diversity in the prevalence of *E. coli* in the examined dairy products (yoghurt, kariesh cheese and cream) (Table 3). The lowest prevalence was for yoghurt, 12% (6 isolates out of 50 samples) and it was significantly lower than those reported for kariesh cheese and cream (p<0.05). Low isolation rate of *E. coli* from yoghurt could be explained on the bases of the organic acid and low molecular weight antimicrobial substance produced by fermenting bacteria in yoghurt such as *Lactobacillus* spp. that showed *in vitro* antimicrobial activity against *E. coli* (Prabhurajeshwar and Chandrakanth, 2019).

Comparing the results of *E. coli* prevalence in yoghurt in the present study with other scholars' result, identical prevalence of *E. coli* (12%) was noted by Okpalugo et al. (2008) in Abuja, Nigeria. On the other hand, Chaleshtori et al. (2017) reported closely matching isolation rate of *E. coli* (10%) from yoghurt in Iran. Higher isolation rates of *E. coli* form yoghurt were also reported 29.5, 44.8 and 88.0 % in Osun, Nigeria; El-Behera, Egypt and Mansoura city, Dakahlia Governorate, Egypt, respectively (El-Ansary, 2014; Abike et al., 2015; Kandil et al., 2018). Higher rates of *E. coli* from yoghurt in different studies could be attributed to the initial load of the milk before processing; usage of mastitic milk (Awadallah et al., 2016), improper sanitation of equipment used during processing; contamination after processing by unhygienic handling, packaging material (Pal *et al*., 2018) or to the storage temperature and time elapsed from manufacturer till sampling (Bachrouri et al., 2006).

On the other hand, the prevalences of *E. coli* in kariesh cheese and cream were 61.8 and 56%, respectively. Higher isolation rates of *E. coli* from kariesh cheese and cream could be attributed to the preparation of these products from mastitic milk (Awadallah et al., 2016) raw milk with high bacterial count, probiotic bacteria are not used in their preparation and also external contamination could occur at one or more points during processing (Deschenes et al., 1996). Non-statistical significant (p>0.05) of *E. coli* isolation rates from kariesh cheese and cream could be attributed to preparation of cream and kariesh cheese from the same milk source. Additionally, previous studies reported a range of 40-75% and 37.5-76.7% isolation rates of *E. coli* from kariesh cheese and cream in different regions in Egypt (Abd El-Tawab et al., 2020; Baraheem et al., 2007; El Nahas et al., 2015; Ibrahim et al., 2019).

Out of 20 *E. coli* isolates represented the dairy products under study 11 serogroups (Table 4) were identified (O25, O26, O27, O55, O78, O86, O114, O124, O125, O128, O148). El-Bagory et al. (2004) identified verotoxigenic *E. coli* O26 from the examined yoghurt samples. Also, Abike et al. (2015) found O26, O55, O86, O114, and O128 serogroups in raw milk, yoghurt and cheese. In a previous study, similar *E. coli* serogroups (O26, O55, and O114) were recovered from kariesh cheese and (O26, O55 and O114) form cream (El Nahas et al., 2015). Many scholars (Scott et al., 2009; Osman et al., 2013; Shehata and Salam, 2012; Awadallah et al., 2016) reported different *E. coli* serogroups either in diarrheic calves, healthy cattle or mastitic milk (O25, O26, O55, O78, 86, O114, O125, O148).

All isolates of *E. coli* recovered from dairy products in the present study were tested for their susceptibility behavior against 11 antimicrobial agents represented different antimicrobial classes of human being and veterinary concern in the region under study. The *in vitro* antimicrobial susceptibility testing revealed diverse susceptibility/resistance behavior of the investigated *E. coli* isolates against the tested antimicrobial agents (Table 5). All 68 tested isolates were 100% sensitive to gentamicin, imipenem, and cefoxitin. On the other hand, the resistance rates against ampicillin, streptomycin, trimethoprim-sulfamethoxazole, cefotaxime, nalidixic acid, tetracycline, and amoxicillin-clavulanic in descending order were 11.8, 10.3, 8.8, 7.4, 5.9, 4.4 and 4.4%, respectively. Side by side, growing of resistance was observed by the intermediate behavior of the investigated isolates against the tested antimicrobial agents. The percentages of the intermediate zones in ascending order were 1.5, 2.9 and 10.3% against trimethoprim-sulfamethoxazole, ciprofloxacin and streptomycin in turn. Additionally, multidrug resistance was noted in seven out of 68 (10.3%) of the inspected *E. coli* isolates.

Results divulged the correlation between the used antimicrobial agents in veterinary medicine and the reporting of resistance in isolates of veterinary origin and vice versa. Cefoxitin, imipenem and ciprofloxacin are of notorious use in medication of large animals and this could explain the results of 0.0% resistance records against these antimicrobial agents. Although the development of intermediate sensitivity behavior against ciprofloxacin and this could be due to the use of ciprofloxacin in broiler industry (Jónsdóttir and Kristinsson, 2008) that can later finds its way to the environmental niche either to humans or other animals. On the other hand, some antimicrobial classes showed higher rates of either resistance or intermediate behavior against the tested *E. coli* isolates likewise, penicillins, aminoglycosides, and tetracyclines, quinolones (first generation) and sulfonamides as these agents are of wide use in veterinary sectors and listed by the OIE as antimicrobial agents of veterinary importance (OIE, 2019). Furthermore, this could explain the prevalence of multidrug resistance amongst the tested isolates (10.3%).

Higher prevalence rates of resistance were reported in Mansoura city, Egypt against streptomycin, nalidixic, cefotaxime, tetracycline, trimethoprim-sulfamethoxazole, ampicillin, ciprofloxacin and gentamicin 100, 80, 60, 60, 60, 40, 40 and 20% in descending order (El-Baz, 2019). Also, Abd El-Tawab et al. (2020) reported 16.7% resistance amongst the *E. coli* tested against tetracycline in El-Gharbia Governorate, Egypt. There is a direct correlation between the abuse of antimicrobials and emergence of resistance amongst bacterial communities (Aly, 2013) and this could expound the metamorphosis in resistance profile of *E. coli* in different areas.

E. coli tempt its pathogenic actions through versatile sets of virulence elements that work in harmony to produce various illnesses in animals and humans. Phenotypic detection of hemolysis revealed the presence of α-hemolysin in 23.5% of the inspected isolates that is an exotoxin produced by *E. coli* and enhances virulence in clinical infections (May et al., 2000). Side by side, genotypic investigation revealed the presence of *ast*A, *eae*A, *stx*1 and *stx*2 genes in variable rates in ten *E. coli* isolates represented those isolated from dairy products under investigation (Figures 1, 2 and 3). All isolated had *ast*A gene, *eae*A was represented in five isolates while *stx*1 and *stx*2 were only noticed in two and one isolates, respectively. EAST1 induced by *ast*A gene associates diarrheagenic *E. coli* in humans and animals and other scholars noted there presence even with non-diarrheagenic *E. coli* (Hinenoya et al., 2014) isolated from healthy cattle and swine. All shiga toxin genes (either *stx*1 or *stx*2) positive isolates were positive also for *eae*A gene. This makes dairy products act as a potential source of STEC for humans as *eae*A genes induce adhesion protein secretion required for intimate adherence of *E. coli* (Blank et al., 2002) that give chance for the cells of *E. coli* to produce shiga toxins to induce either watery or bloody diarrhea and may lead to a lethal disease such as HC, TTP and HUS (Khalid and Anderoli, 2018).

Researchers in different regions reported *ast*A, *eae*A, *stx*1 and *stx*2 genes in *E. coli* isolated from dairy products with variable prevalence rates (Elafify et al., 2020; Dehkordi et al., 2014) which could be attributed to the difference in the circulating serotypes in every study area.

CONCLUSIONS AND RECOMMENDATIONS

The present study points to yoghurt, kariesh cheese and cream as serious potential source of various *E. coli* pathotypes harboring virulence factors able to induce lethal diseases in humans. Moreover, multidrug resistant strains of *E. coli* that even if non-pathogenic will participate in establishing resistance in gastrointestinal tract bacterial community and environment. So, there is a fundamental need to follow the implementation of both good hygiene and manufacturing practices as well as application of strict hazards analysis and critical control point in dairy products industry for the sake of human safety.

AUTHOR'S CONTRIBUTION

All authors contributed equally.

Conflict of interest

The authors have declared no conflict of interest.

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