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Incidence and molecular characterization of *Escherichia coli* in some dairy products

Ekbal M.A. Ibrahim[1], Hend A. Elbarbary[1], Nahla A. Shawky[2] and Islam A. El-Sebay[3]

[1] *Department of Food Hygiene, Faculty of Veterinary Medicine, Benha University, Egypt.*

[2]*Animal Health Research Institute, Shebin El-Kom Branch, Egypt.*

[3] *Food Quality Control, Minoufiya University, Egypt.*

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ABSTRACT

Escherichia coli (*E. coli)* is one of foodborne pathogens associated with several cases of human sickness. The present study was undertaken to estimate the incidence of *E. coli* in total of 120 random samples of some dairy products; Raw milk, Kareish cheese, Damietta cheese and Cream (30 samples each); randomly collected from local markets, farmer vendors and supermarkets at different localities in Nile Delta region; Minoufiya Governorate, Egypt. Confirmation of the isolated *E. coli* serovars was performed using a series of biochemical tests, serological and PCR identification. The results revealed that 70.8% of the examined samples were *E. coli* positive. Twelve serogroups could be identified serologically including *E. coli* O26 : H11, O44 : H18, O55 : H7, O91 : H21, O111 : H2, O119 : H6, O121 : H7, O124, O127 : H6, O128 : H2, O146 : H21, O153 : H2. Finally, the results of Multiplex PCR with specific primers for detection of *Stx1*, *Stx2* and *eaeA* genes, revealed that the isolates of *E. coli* O26 : H11, O91 : H21, O127 : H6, , O111 : H2, and O153 : H2 had *Stx1* and *Stx2* genes, while, *E. coli* O119 : H6, and O128 : H2 had only *Stx1*.Also O44 : H18, O55 : H7, O121 : H7, and O146 : H21had only *Stx2*. Concerning the *eaeA* gene, *E. coli* O111 and O26 isolates possessed this gene.

1. INTRODUCTION

Escherichia coli (*E. coli*) is one of normal inhabitant microorganisms of large intestine in human and warm-blooded animals. The main source of *E. coli* in raw milk and milk products is fecal contamination together with poor hygienic practices (Garbaj *et al*., 2016; Lara *et al*., 2016). However, certain serotypes of *E. coli* have acquired some virulence-associated genes that enable them to cause intestinal or extra-intestinal disease (Hodson *et al.*, 2017).

E. coli is generally used as an indicator of direct or indirect fecal contamination and the possible presence of enteric pathogens in milk and dairy products (Kornaki and Johnson, 2001). These serotypes that cause enteric infections are generally called diarrheagenic *E. coli* strains, and their pathogenesis is associated with a number of virulence attributes, which vary according to pathotype. Currently, diarrheagenic *E. coli* strains are classified into six main pathotypes based on their distinct virulence determinants and pathogenic features, including enteropathogenic *E. coli* (EPEC), enterotoxigenic *E. coli* (ETEC), enterohemorrhagic *E. coli* (EHEC), shiga toxin-producing *E. coli* (STEC), enteroinvasive *E. coli* (EIEC), enteroaggregative *E. coli* (EAEC) and diffusively adherent *E. coli* (DAEC) (Ruttler *et al*., 2006; Xia *et al*., 2010).

A large variety of serotypes have been isolated from patients with gastrointestinal disease and many of these serotypes as well as others have been isolated from animals. The serotypes associated with illness in humans include members of O serogroups 26, 91, 103, 111, 113, 121, 145 and 157 (Karmali *et al*., 2010; Pizarro *et al*., 2014).

This study was undertaken to investigate the prevalence of enteropathogenic *E. coli* in raw milk and some dairy products, and the incidence of some virulence-associated genes in the isolated *E. coli* serotypes was examined using polymerase chain reaction (PCR).

2. MATERIAL AND METHODS

2.1. Collection of samples

One hundred and twenty samples of some dairy products; Raw milk, Kareish cheese, Damietta cheese and Cream (30 of each); were randomly collected from local markets, farmer vendors and supermarkets at different localities in Nile Delta region; Minoufiya Governorate, Egypt. The collected samples were transferred directly to the laboratory in an ice box under complete aseptic conditions. The samples were immediately examined bacteriologically for the incidence of *E. coli* and detection of the isolated *E. coli* serovars using serology and PCR techniques.

* Corresponding author: **Islam A. El-Sebay.** Food Quality Control, Minoufiya University, Egypt.

2.2. Preparation of samples
The samples were prepared according to the technique recommended by APHA (2004). Ten ml from each well mixed raw milk, cream samples and Twenty-five gm of the examined cheese samples were transferred to blender jar and homogenized with 90 and 225 ml of tryptone soya broth (TSB), respectively under complete aseptic conditions. Stomacher-Blender (Seward Medical, London, England) was used to homogenize cheese samples.

2.3. Preparation of serial dilutions (APHA, 2004)
Eleven gm of well mixed samples were aseptically weighed and directly transferred into sterile flask contain 99 ml of sterile peptone water (1%) then thoroughly mixed to prepare a dilution of 1/10 from which decimal dilutions were prepared.

2.4. Isolation and identification of E. coli (APHA, 2004)
One ml of the previous homogenate was inoculated separately into MacConkey broth tubes supplemented with inverted Durham's tubes. The inoculated and control tubes were incubated at 37 ºC for 24-48 hours. Tubes showing acid and gas production were considered positive for *coliforms*, and the results were recorded.
A loopful from each positive MacConkey broth tube was inoculated into another MacConkey broth tube and incubated at 44±0.5 ºC for 48 hours, after that a loopful from each positive tube (with gas production) was streaked onto Eosin Methylene Blue (EMB) agar plates, then incubated at 37 ºC for 24 hours. Typical colonies of *E. coli* appeared greenish metallic with dark purple center. Suspected colonies were picked up and transferred to nutrient agar slopes and then incubated at 37 ºC for 24 hours. The purified colonies were subjected for further morphological, biochemical and serological examination.

2.5. Identification of the isolates according to *APHA (2004)*
2.5.1. Microscopical examination (Cruickshank et al., 1975)
Films were made from the pure culture of isolated organisms stained by Gram's stain *and* examined microscopically. Gram-negative coccobacilli, medium size rods and non-sporulating were recorded.
2.5.2. Motility test (ICMSF, 2006)
E. coli showed positive reaction through spreading growth around stabbing line. *E. coli* showed negative result.
2.5.3. Biochemical tests (Mac Fadin, 2000).
2.5.4. Serological identification Kok et al. (1996)
Isolates proved biochemically to be *E. coli* microorganisms were subjected to serological identification by using rapid diagnostic *E. coli* antisera sets (Denka Seiken Co., Japan). For the determination of enteropathogenic types.
2.5.5. Polymerase chain reaction (PCR) (Hu et al., 2011 and Dipineto et al., 2006) for confirmation of isolated strains and for detection of shiga toxin1 (stx1 gene) shiga toxin2 (stx2 gene) and intimine (eaeA).
2.5.5.1. Molecular characterization of virulence associated genes
DNA Extraction using QIA amp kit (Shah et al., 2009). After overnight culture on nutrient agar plates, one or two colonies were suspended in 20 ml of sterile distilled water, and the suspension was then heated at 100 ºC for 20 min. Accurately, 50-200 µl of the culture were placed in Eppendorf tube. The PCR mixture was prepared by adding 10 µl Emerald Amp GT PCR master mix (Takara, Japan), 3 µl PCR grade water, 1 µl Forward primer (20 pmol), 1 µl Reverse primer (20 pmol), 6 µl Template DNA to a total volume of 25 µl. The investigated virulence associated genes (*stx*1, *stx*2 and *eae*A) were amplified using the specific primers.

Primer sequences and predicted lengths of multiplex-PCR amplification products:

Target gene	Oligonucleotide sequence (5′ → 3′)	Product size (bp)	References
stx1 (F)	5′ ACACTGGATGATCTCAGTGG '3	614	Dhanashree and Mallya (2008)
Stx1 (R)	5′ CTGAATCCCCCTCCATTATG '3		
Stx2 (F)	5′ CCATGACAACGGACAGCAGTT '3	779	
Stx2 (R)	5′ CCTGTCAACTGAGCAGCACTTTG '3		
eaeA (F)	5′ GTGGCGAATACTGGCGAGACT '3	890	Mazaheri et al. (2014)
eaeA (R)	5′ CCCCATTCTTTTTCACCGTCG '3		

3. RESULTS

Data in table (1) revealed that the incidence of *E. coli* was 27 (90%), 22 (73.3%), 13 (43.3%) and 23 (76.7 %) for the examined Raw milk, Kareish cheese, Damietta cheese and Cream samples, respectively.

Table 1 Incidence of *E. coli* in the examined dairy products (n=30).

Examined samples	Positive samples No.	%
Raw milk	27	90%
Kareish cheese	22	73.3%
Damietta cheese	13	43.3%
Cream	23	76.7%
Total	85	70.8%

Inspection of table (2) revealed that the serologically identified *E. coli* isolates in the examined dairy samples were O26 : H11, O44 : H18, O55 : H7, O91 : H21, O111 : H2 , O119 : H6, O121 : H7, O124, O127 : H6, O128 : H2, O146 : H21, and O153 : H2 .
The isolates *E. coli* O26 : H11, O91 : H21, O127 : H6, , O111 : H2, and O153 : H2 had Stx1 and Stx2 genes while, *E. coli* O119 : H6, and O128 : H2 had only Stx1.Also O44 : H18, O55 : H7, O121 : H7 and O146 : H21 had only Stx2. Concerning the eaeA gene, *E. coli* O111 and O26 isolates possessed this gene (Table 3).

4. DISCUSSION

E. coli can get access to milk and its products, which is one of the main inhabitant microorganisms of the intestinal tract of most mammalian species, including humans and birds. Most of *E. coli* are harmless, but some were known as pathogenic bacteria causing severe intestinal and extra intestinal diseases in man (Kaper *et al., 2004). E. coli* is recognized as a serious foodborne pathogen and associated with numerous outbreaks in the UK, Japan, and USA (Scotter et al., 2000).
The data presented in table (1) showed that 27 out of 30 Raw milk samples (90%) were positive for *E. coli*. These results were in agreement with those postulated by Gamal et al. (2015), who revealed that (80%) of examined raw milk samples were *E. coli* positive, and higher than those reported by Lingathurai and Vellathurai (2013), El nahas et al. (2015), Elbagory et al. (2015), Saba et al. (2015), Ombarak et al. (2016), and Disassa et al. (2017), who reported that

(70%), (55%), (25%), (49.3%), (76.4%) and (39.1%) of examined raw milk samples were *E. coli* positive.

Our results revealed that the examined raw milk samples were highly contaminated by *E. coli*, this may be due to fecal contamination of milk during or after milking through improper washing and disinfection of the udder or contact with contaminated milking pails and utensils.

Concerning Kareish and Damietta cheese samples, the results obtained revealed that incidence of *E. coli* were 22 (73.3%) and 13 (43.3%) of examined Kareish and Damietta cheese samples, respectively. Our results agreed with those postulated by Ombarak et al (2016), who found that *E. coli* could be detected in percentage of (74.5%) of examined Kareish cheese samples. Lower results were recorded by Gamal et al (2015), El nahas et al (2015) and Elbagory et al (2015) in percentage of (33%), (50%) and (37.5%) of the examined Kareish cheese samples respectively. Higher results were recorded by El-Kosi (2001) and Mohamed (2015), who found that 100% of examined Kareish cheese samples were contaminated with *E. coli*. Our results failed to comply with the Egyptian Standards (2005) which pointed out that the Kareish cheese must be free from *E. coli*.

For Damietta cheese, the obtained results were higher than those mentioned by El-Bessery (2006), Gamal et al. (2015) and Elbagory et al. (2015), who could detect *E. coli* with percentage of (10%), (20%) and (15%) of examined Damietta cheese samples, respectively. Presence of *E. coli* in the examined Damietta cheese samples may be due to the use of unpasteurized milk for production of cheese or contamination after pasteurization during production and handling of cheese. Furthermore, the way of selling some Damietta cheese (not properly packed) could be a cause of contamination of cheese with *E. coli* after production (Saad et al., 2012).

For Cream samples, 23 out of 30 samples contained *E. coli*. Our results were higher than those recorded by El-Essawy and Riad (1990), Abd El-Hameid (2013) and El nahas et al (2015), who found that *E. coli* could be detected in (16%), (53.3%) and (47.5%) of examined cream samples. Such results may be due to manufacturing of cream by gravity or farmer method that make cream more susceptible for microbial contamination (Godefay and Molla, 2000).

Table 2 Serological identification of the isolated *E. coli* strains from the examined samples

E. Coli Serotype	Strains grouping *	Raw milk		Kareish cheese		Damietta cheese		Cream		Total	
		No.	%	No.	%	No.	%	No.	%	No	%
O128 : H2	ETEC	3	10	4	13.33	2	6.66	2	6.66	11	36.66
O26 : H11	EHEC	9	30	3	10	4	13.33	4	13.33	20	66.66
O121 : H7	EHEC							1	3.33	1	3.33
O44 : H18	EPEC			2	6.66	1	3.33			3	10
O91 : H21	EHEC	3	10			1	3.33	1	3.33	5	16.66
O119 : H6	EPEC			1	3.33			2	6.66	3	10
O127 : H6	ETEC			2	6.66	1	3.33			3	10
O111 : H2	EHEC	5	16.66	4	13.33	2	6.66	2	6.66	13	43.33
O55 : H7	EPEC	1	3.33			1	3.33			2	6.66
O124	EIEC			1	3.33	1	3.33	1	3.33	3	10
O146:H21	EPEC			1	3.33	1	3.33	2	6.66	4	13.33
O153:H2	EPEC	1	3.33					1	3.33	2	6.66
Total		23	76.66	18	60	12	40	17	56.66	70	58.33

Strains grouping according to ICMSF (2006). EHEC: Entrohemorregic E. coli, EPEC: Enteropathogenic E. coli, ETEC: Enterotoxigenic E. coli, EIEC: Enteroinvasive E. coli

Table 3 Distribution of *stx1* (614 bp), *stx2* (779 bp) and *eaeA* (890 bp) virulence genes in *E. coli* isolates from examined dairy products

E. coli serogroup	*stx1*	*stx2*	*eaeA*
O26 : H11	+	+	+
O44 : H18	-	+	-
O55 : H7	-	+	-
O91 : H21	+	+	-
O111 : H2	+	+	+
O119 : H6	+	-	-
O121 : H7	-	+	-
O124	-	-	-
O127 : H6	+	+	-
O128 : H2	+	-	-
O146 : H21	-	+	-
O153 : H2	+	+	-

eaeA: intimin gene. *stx1*: Shiga- toxin 1 gene. *stx2*: Shiga- toxin 2 gene

Data in table (2) illustrated that the incidence of serologically identified *E. coli* isolated from the examined samples was 12 (22%) represented as O26 : H11, O44 : H18, O55 : H7, O91 : H21, O111 : H2 , O119 : H6, O121 : H7, O124, O127 : H6, O128 : H2, O146 : H21, and O153 : H2 . Nearly similar isolation of E. coli was recorded by Al-Ashmawy (2004), El-Bagory and Hammad (2004), Paneto et al. (2007), Altalhi and Hassan (2009), Virpari et al (2013), Elbagory et al. (2015), and El nahas et al (2015).

DebRoy and Maddox (2001) stated that *E. coli* strains can cause considerable losses of neonatal animals where Enterotoxigenic strains attach to the brush border membrane of the jejunum and ileum and cause watery yellow, white to grey diarrhea in neonatal animals. *Enteropathogenic* strains of *E. coli* form lesions that destroy the brush border of the small intestine and form pedestal structures so that the bacteria remain in contact with the cells and cause chronic, mucoid diarrhea, as they colonize the colon and causes necrosis of the villi; diarrhea is mucoid, sometimes hemorrhagic, and seldom fatal but often recurrent even with treatment, resulting in dehydration and reduced growth. *Necrotoxigenic* strains produce a toxin called cytotoxic necrotizing factor (CNF) and although not much is known about the pathogenesis.

Molecular characterization of virulence associated genes was carried out on the typical 12 isolates (Table 3). The results of molecular identification of stx1, stx2 and eaeA virulence associated genes revealed that the isolates *E. coli* O26 : H11, O91 : H21, O127 : H6, , O111 : H2, and O153 :

H2 had Stx1 and Stx2 genes, while, *E. coli* O119 : H6, and O128 : H2 had only Stx1.Also O44 : H18, O55 : H7, O121 : H7 and O146 : H21had only Stx2. Concerning the eaeA gene, *E. coli* O111 and O26 isolates possessed this gene. Our results were in agreement with those of Elhadidy and Mohammed (2012). The pathogenicity of STEC is attributed to the production of stx1 and stx2 as verocytotoxin (Hessain et al., 2015). The gene stx2 is the most important virulence factor and most of hemolytic-uremic syndrome cases in humans are caused by STEC strains harboring stx2 gene (Elhadidy and Mohammed , 2012). The eae gene is an accessory virulence factor for STEC that is thought to enhance the virulence of STEC, while some STEC strains not harboring eae gene have been shown to cause human illnesses (Neill, 1997; Kruger and Lucchesi, 2015). Further, Douellou et al. (2017) demonstrated that the virulence gene profiles of dairy products and human STEC strains were similar, this explain the involvement of pathogenic STEC in sporadic cases and disease outbreaks in human

5. CONCLUSION

The results of this study declared that the examined raw milk and dairy products may be a possible source for transmission of enteropathogenic *E. coli* to human. The isolated *E. coli* serotypes harbored various virulence-associated genes, in particular those responsible for hemorrhage and toxin production. Accordingly, adoption of strict hygienic measures should be followed.

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