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# *Escherichia coli* that Produces Shiga Toxins: Prevalence and Possible Risk in Soft Cheese

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**Abstract**

THOUGH soft cheese is vital nutrition source for many people, yet if made from unpasteurized milk, it poses a concern to public health e.g *Escherichia coli* that can produce Shiga toxins (STEC). In this study totally, 150 soft cheese (50 Karish, 50 Tallaga, 40 processed and 10 feta cheeses) samples were collected indiscriminately from several areas in El-Gharbia Governorate and tested for STEC. By culturing on EMB, *E.coli* were detected in (18/50) 36%, (12/50) 24%, (1/40) 2.5% and none (0/10) of the examined samples, respectively. One hundred suspected isolates were examined biochemically; thirteen identified isolates were serotyped: of them 11 strains were confirmed as pathogenic STEC serotypes (O78:K80, O44:K74, O114:K90, O124:K63, O103:K- and O145:K-). Seven strains that obtained from Karish and Tallaga cheese were tested for virulence factors as Shiga toxins (stx1 and stx2) and intimin (eaeA) genes using polymerase chain reaction (PCR). Three out of the seven (42.9%) strains have stx1 and eaeA genes, 1/7 (14.3%) carry stx2 and eaeA genes, 1/7 (14.3%) carry stx1, stx2 and eaeA genes while 2/7 (28.6%) carry only eaeA gene. STEC strains that have shiga toxin with adherence genes are considered to pose high risk of illness that necessitates strict hygienic measures enforcement during soft cheese production.

**Keywords:** *E. coli*, Shiga toxins, virulence genes, soft cheese.

## Introduction

Soft cheese is rich in vital nutrients such as fatty acids, amino acids, bioactive peptides that decrease hypertension, vitamins and minerals especially calcium that has positive effects on disorders like osteoporosis, obesity and dental caries [1]. Though, soft cheese is a vital nutritive source for several people, but it may be a dangerous source for infections with pathogenic *E. coli* that able to produce Shiga toxins (STEC), especially Karish and Tallaga cheese [2].

Globally, there is increase in the spread of diseases transmitted via food consumption and caused by STEC [3]. Food and Agriculture Organization of the United Nations cited that about 18 x 106 tons of cheese are produced worldwide each year (http://faostat.fao.org) [4].

As cheese, mainly freshly eaten soft types may elucidate hazard for consumers as a result of foodborne pathogens transition so cheese made in street, farmers' home or in unlicensed factories is considered unsafe to be consumed [5].

The traditional production of raw milk cheese eg. Karish (famous Egyptian unripen, fresh soft) cheese, which produced by farmers at small-scale in rural areas, then sold to the urban consumers by agri-food markets. Although this practice is a widely spread in many countries [6], yet, the resulted products is likely to be a vehicle for various harmful pathogens, including *E. coli* [7 & 8].

STEC is among the most significant groups of foodborne pathogen causes diseases ranging from uncomplicated watery and/or bloody diarrhoea to serious illness as hemorrhagic colitis (HC) and potentially lethal consequence like hemolytic uremic

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syndrome (HUS) [9], the main reason of acute renal failure in children [10].

Actually, the criteria utilized to identify STEC strains that can infect human is serogroup [11]. Worldwide *E. coli* O157:H7 is the most frequently reported cause of extreme STEC sickness and outbreaks, yet there were evidence connecting the "top 6" non-O157 STEC serogroups (e.g., O26, O45, O103, O111, O121, and O145) to serious infections and outbreaks. Furthermore, else 250 STEC serotypes have relation with human diseases [12].

Shiga toxin–producing E. coli (STEC), also known as verotoxin-producing E. coli (VTEC) or enterohaemorrhagic E. coli (EHEC), have been known as a group of highly pathogenic E. coli strains producing one or more Shiga toxins [13], that inhibit protein synthesis in the endothelial and other cells, moreover damage of vascular endothelium [14]. There are four variants belong to Stx1 (a, c, d, and e) and twelve variants of Stx2 (a-i) among the primary virulence factors virulence Stx1 and Stx2 [15].

Thus, Shiga toxin types 1, 2 and eaeA gene (which encodes intimin and permits the close attachment of E.coli to the intestinal lining epithelia), are the primary virulence genes responsible for the pathogenicity of STEC [16].

In sight of these facts, the current investigation objective is to detect, identify and molecularly characterize STEC in soft cheese samples gathered from several locations within EL-Gharbia Governorate and outline the possible public health hazard of them.

## Material and Methods

*Sample Collection*

Over the duration starting from March to July 2023, totally (150) soft cheese samples were gathered from diverse areas in EL-Gharbia Governorate, Egypt. Fifty Karish (of which (25) were from farmer-houses using pottery containers and (25) from different markets), 50 Tallaga, 40 processed and 10 feta cheese samples were gathered in aseptic condition from various markets in sterile containers or in their packaging, maintained in icebox at $4^{o}$C and promptly delivered to the lab for testing at once.

According to EOSQ [17], Karish cheese is soft, un-ripened cheese made from skim milk and consumed fresh whereas Talaga cheese is represented as soft cheese made from pasteurized milk that is refrigerated after being preserved in brine [18].

*Sample preparation and pre-enrichment*

A stomacher was used to homogenize 25 g/ sample with 225 ml of 1% buffered peptone water (BPW, Oxoid, Hampshire, UK) in polyethylene bag for two minutes as described by [19]. McConkey broth medium (90 ml) (HiMedia, Maharashtra, India) were inoculated with 10 ml cultured BPW and kept for 18 to 24 h. at 37°C.[20]

*E. coli isolation of in solid media*

Since few numbers of bacteria (STEC) can cause disease in humans, so using enrichment media is necessary to find these organisms in the samples under examination [21]. After streaking of a Loopful of enriched broth on Eosin Methylene Blue (EMB, HiMedia, India), it was incubated for 18 to 24 h. at 37°C. From plates exhibiting typical colonies with greenish metallic sheen [22], four to five colonies were selected and inoculated in semisolid nutrient broth media (pure culture) and kept for 18 to 24 h at $37^{o}$C then stored at 4°C.

*Biochemical Examination*

One hundred suspected isolates were identified biochemically according to [23] which include IMViC, triple sugar iron (TSI) and urease tests. Thirteen verified *E. coli* isolates were kept at –80°C in nutrient broth including 30% sterile glycerol [24] .

*Serological identification E. coli*

Based on the Anti-coli reagents approach (SIFIN Co., Germany) for serotyping of the Enteropathogenic types depend on (O) somatic and (K) capsular antigens, using slide agglutination test, thirteen verified *E. coli* isolates were typed using of standard polyvalent and monovalent *E. coli* antisera. This test was performed at the Animal Health Research institute, Agriculture Research center, Dokki.

*Molecular characterization of serotyped enterohaemorrhagic E. coli strains*

*DNA Extraction*

Using some modifications from the manufacturer's recommendations. QIAamp DNA Mini kit (Qiagen, Germany, GmbH) was utilized to extract DNA from seven *E. coli* isolates. Two hundred microliters suspension of each sample was treated for 10 minutes at 56°C with 10 µl of proteinase K and 200 µl of lysis buffer. Two hundreds µl of hundred percentage ethanol was added to the lysate following incubation. Then, the samples were washed and centrifuged following up the manufacturer's instructions. Then use 100 µl elution buffer given in the kit was added to elute nuclic acid [25].

Genes responsible for production of Shiga toxins (stx1 and stx2) and intimin (eaeA) were amplified by using Oligonucleotide primer sequences from (Metabion, Germany) as shown in Table 1.

*Statistical Analysis*

Differences in positive and negative cheese samples for STEC, according to source of collection of karish cheese, were statistically analyzed using the Pearson's chi-square ($\chi$ 2 ) test. The results were considered statistically significant for $p \leq 0.05$.[28].

**Results and Discussion**

From food hygiene and safety point of view, strains of *E.coli* that produce shiga toxins (Stx) represent public health risk as they can seriously affect humans when they move down the food chain from their animal source (mostly faeces to milk or meat) [29], and the detection of EHEC through their shiga toxins and eae genes is substantial to ensure food safety[30].

In this study, based on morphological character of colonies on EMB agar, 31(20.67%) out of the examined (150) soft cheese samples are *E. coli* positive. Of them 18(36%), 12(24%), 1(2.5%) of Karish, Tallaga and Processed cheese samples respectively were positive, while all the examined ten feta cheese samples were negative (Table, 2). The difference between detection percentages in different cheese samples is attributed to variation in efficacy of sanitary precautions used throughout processing, handling and distribution of cheese [31]. Lower percentage of *E.* coli recorded by previous researchers for soft cheese samples as (16%) for Karish cheese [32] and (30 %) Karish and (12%) Tallaga [31] while higher results (73.3% and 54%) were reported by [34&20] for Karish also for Tallaga (32%) [20]. For processed cheese, higher result was recorded by [35] also by [36] for Feta (8%) cheese.

From 31 positive soft cheese samples, 100 isolates were obtained, of them 61, 38 and 1 were obtained from Karish, Tallaga and Processed cheese, respectively, and then examined biochemically. Only 13 isolates were confirmed to be *E. coli* and only 8 (5.33%) samples were confirmed to be contaminated by *E.* coli (Table, 2).

Out of the examined 13 serolgically identified isolates, 11(84.62%) were serotyped as Enterovirulent *E. coli* strains and 2 isolates non *E.coli* (Table 3). Seven strains were belonging to Karish cheese samples of them 6(6/8, 75%) obtained from farmer houses and one (1/1, 100%) from markets Karish cheese samples. In addition to 4 isolates obtained from Tallaga cheese also serotyped as EPEC. Thus among *E. coli* contaminated samples recorded by culture method only 7/31(22.58) samples proved by serological test to contain Enterovirulent strains, this record is nearly similar to that (19.48%) reported by [37] who detected 8 EPEC serogroups from samples out of 76 contaminated soft cheese. Our results showed two serotypes in Tallaga cheese (Table 3) while [20] didn't find any serotypes.

Depend on somatic (O) and capsular (K) antigens 6 different serogroups of *E. coli* were identified : O78:K80, O44:K74, O114:K90, O124:K63, O103:K- and O145:K- and one untyped *E. coli* while O157 wasn't recovered in this research (Table 3). These results came in agreement with those of [38] in which, they reported serotypes O111:H4, O124: H-, O127:H6, and O55:H7 in soft cheese. In addition to *E. coli* serotypes O157, O78, O103, O118, O124, O145, and O164 were detected in soft cheese [39]. Also, [40] recovered *E. coli* serotypes O26: H11, O124, O114, O15, O125: H21 and O111: H2 and one strain Untyped from Karish cheese. The results obtained by [41] for Karish cheese were serotyped as O86, O121, O157, O119, O142 and O128.

Regarding source of Karish cheese samples, results in (Table 2) showed that samples obtained from farmer houses recorded significance higher contamination percentage (52%) than counterpart samples collected from markets (20%). As at the farm cheese are liable for contamination from unclean utensils, hands and moreover it is sold unpacked. After serological identification, the percentages of confirmed positive samples in two sources (12% in farmer house and 4% in markets samples) of karish cheese showed no significant difference when analyzed bio statistically by the Chi-Square ($\chi$ 2) (test for independence) for $p \leq 0.05$ (Table 3).

Detection of EPEC stains in 4/50 (8%) and 3/50 (6%) of examined Karish and Tallaga cheese samples may be related to the fact that cheese is made from raw milk or due to using contaminated utensils and mishandling especially these products not efficiently packed and are liable to contamination during selling [42].

STEC strains with the same serotypes could not have the same virulence factors or evoke similar risk since numerous STEC virulence genes are mobile and easily lost. Consequently, the identification of these genes is indispensable to appreciate the health risk posed by STEC strains as serotypes data alone is insufficient [43].

Though Shiga toxins (stxs) virulence factors are important for the pathogenicity and severity of STEC [44] but they alone without adherence is considered insufficient to induce severe illness [43]. A further STEC virulence gene that is crucial in enhancing the pathogenicity is the intimin (eaeA) gene [32]. Despite the fact that certain STEC strains that cause illness in humans lack it [45].

Seven strains were examined by multiplex PCR technique in our study, intimin(eaeA) gene is recovered in all (100%) examined strains, stx1 is detected in 4(57.14%) and stx2 in only 2(28.6%) with

absence of stx1 and 2 in two serotypes (Table, 4 & Fig. 1). A Lower incidence of stx1, stx2 was cited by [46]. A higher incidence was found by [47] who reported that whereas 21% of *E. coli* isolates had eaeA gene, all recovered isolates (100%) had stx1 and stx2 genes. In contrast, [48] found no isolate with the eaeA gene, which contradicts our findings. The eaeA gene is required for the bacteria to adhere to epithelial cells [49]. The stx1 gene wasn't detected in all isolates from Karish and Tallaga cheese which examined by [50]. While [51] recorded that stx2 is more frequent than stx1 in the examined *E.coli* isolates.

Our study shows that 4/50 (8%) and 3/50 (6%) of the examined Karish and Tallaga cheese samples were contaminated with Enteropathogenic *E. coli*. Consequently, not meet the Egyptian requirements that stated that these products had to be pathogens free [17 & 18]. So this hazard must be controlled and verify that HACCP are being applied [52].

## Conclusion

Our findings revealed that soft cheese sold in El-Gharbia Governorate represent potential vehicle for human pathogens especially the detected STEC in the examined samples which indicate unhygienic processing and handling. That necessitates using high quality raw milk and efficient pasteurized milk for soft cheese manufacture, strict regulatory measures throughout processing, handling and marketing, also selling should be restricted to healthy authorized shops that apply hygienic measures to avoid public health risk of consumers.

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*Conflict of interest:*

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**TABLE 1. Primer sequences that were utilized to identify virulence genes of *E. coli***

| Bacteria | Gene | Sequence | Product of Amplification | Reference |
|---|---|---|---|---|
| *E. coli* | stx1 | ACACTGGATGATCTCAGTGG<br>CTGAATCCCCCTCCATTATG | 614 bp | [26] |
| | stx2 | CCATGACAACGGACAGCAGTT<br>CCTGTCAACTGAGCAGCACTTTG | 779 bp | |
| | eaeA | ATGCTTAGTGCTGGTTTAGG<br>GCCTTCATCATTTCGCTTTC | 248 bp | [27] |

**TABLE 2. *E. coli prevalence* in soft cheese samples examined by cultural method and biochemical tests**

| Type and number (n) of cheese samples | | Culture on EMB agar | Biochemical Examination | |
|---|---|---|---|---|
| | | Number of positive samples of *E. coli* (%)[1] | Number of positive isolates of *E. coli* (%)[2] | Number of positive samples of *E. coli* (%)[3] |
| Karish (50) | Farmer-house (25) | 13 (52%) * | 8/47 (17.02%) | 4 (16%) |
| | Markets (25) | 5 (20%) * | 1/14 (7.14%) | 1 (4%) |
| Tallaga (50) | | 12 (24%) | 4/38 (10.53%) | 3 (6%) |
| Processed (40) | | 1 (2.5%) | 0/1 (0%) | 0 |
| Feta (10) | | 0 (0%) | 0% | 0 |
| Total (150) | | 31 (20.67%) | 13/100 (13%) | 8 (5.33%) |

**(%)[1]**= percentages were calculated according to the number of examined samples of each type
**(%)[2]**= percentages were calculated according to the number of examined isolates
**(%)[3]**= percentages were calculated according to the number of examined samples of each type
*Significant for $p \leq 0.05$ by the Chi-Square ($\chi$ 2) (test for independence)

**TABLE 3. Serotyping of confirmed *E. coli* isolates obtained from soft cheese samples**

| Source of the examined isolates (number) | | Number of *E. coli* serotyped isolates (%)[1] | Number of positive samples with *E. coli* serotypes (%)[2] | Serotypes *E. coli* (number of isolates) |
|---|---|---|---|---|
| Karish (9) | Farmer-House (8) | 6 (75%) | 3/25 (12%) * | O114:K90(1), O124:K63 (2), O103:K-(2), O145:K- (1) |
| | Markets (1) | 1 (100%) | 1/25 (4%) * | O44:K74 (1) |
| Tallaga (4) | | 4 (100%) | 3/50 (6%) | O78:K80 (2), O44:K74 (1), untyped (1) |
| Total (13) | | 11 (84.62%) | 7/150 (4.67%) | 6 serogroups |

(%)[1]= percentages were calculated according to the number of examined *E. coli* isolates
(%)[2]= percentages were calculated according to the number of examined samples of each type
*Non significant for $p \leq 0.05$ by the Chi-Square ($\chi$ 2) (test for independence)

**TABLE 4. Profile of virulence genes in STEC strains obtained from the examined soft cheese samples**

| Detected virulence gene | Number of *E. coli* strain (%)* | *E. coli* serotypes (number of isolates) |
|---|---|---|
| (*stx1*) and intimin(eaeA) | 3/7 (42.9%) | O78:K80 (1), O145:K- (1), untyped (1) |
| (*stx2*) and intimin(eaeA) | 1/7 (14.3%) | O44:K74 (1) |
| *Stx1, stx2 and* intimin (*eaeA*) | 1/7 (14.3%) | O114:K90 (1) |
| Intimin (*eaeA*) | 2/7 (28.6%) | O103: K- (1), O124:K63 (1) |

* Percentages were calculated according to the number of examined strains (7)

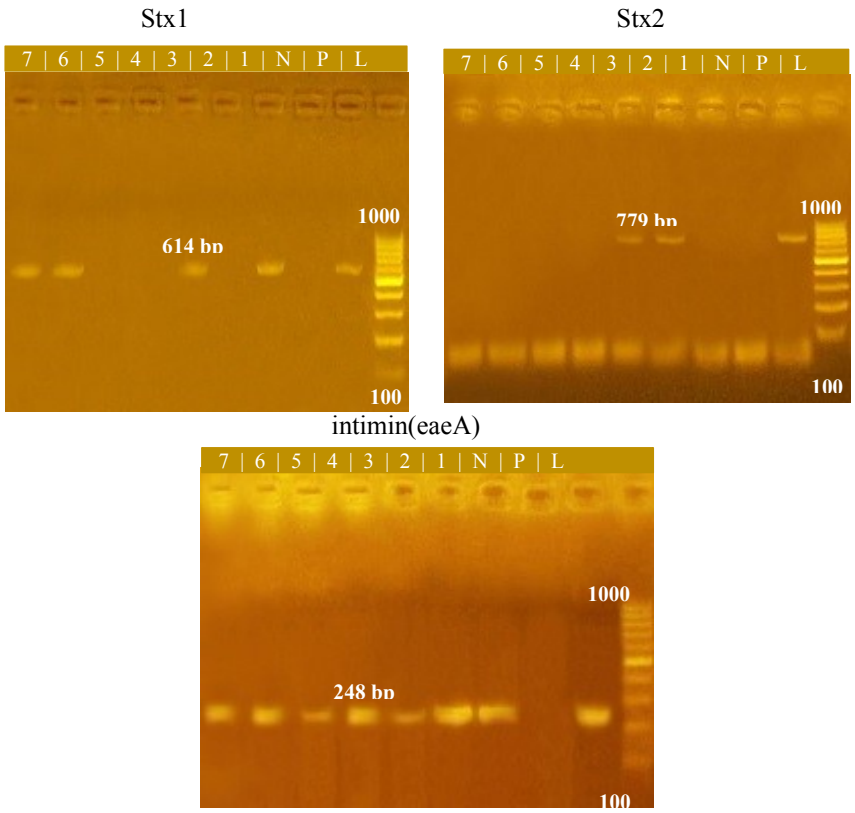


**Fig. 1. Multiplex PCR of the virulence genes stx1, stx2, and, eaeA (614, 779, and 248 bp, respectively) using Agarose gel electrophoresis to characterize enteropathogenic *E. coli*. Lane L: 100 bp ladder DNA marker. Lane P+: control positive for stx1, stx2, and eaeA. Lane N−: negative control for stx1, stx2, and, eaeA. Lanes 1, 3,6 and 7: positive isolates for stx1. Lanes and 3: positive isolates for stx2. Lanes 1, 2, 3,4,5,6 and 7: positive isolates for eaeA.**

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## بكتيريا الإيشريشيا كولاى المنتجة لسم الشيجا: الأنتشار والمخاطر المحتملة في الجبن الطري

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**الملخص**

بالرغم من ان الجبن الطري مصدرًا غذائيًا حيويًا للعديد من الأشخاص، ولكن إذا تم إنتاجه من حليب غير مبستر يمثل خطر الإصابة بمسببات الأمراض المنقولة بالغذاء مثل الإيشريشيا كولاى المنتجة لسموم الشيجا (STEC). في هذه الدراسة، تم تجميع 150 عينة من الجبن الطري (50 قريش، 50 جبنة تلاجة، 40 جبنة مطبوخة و10 جبنة فيتا) بشكل عشوائي من مناطق مختلفة في محافظة الغربية وتم اختبارها للكشف عن STEC. من خلال الزراعة على EMB، تم الكشف عن الإيشريشيا كولاى في (50/18)36%، (50/12)24%، (40/1)2.5% ولا شيء (10/0) من عينات الجبن القريش والتلاجة والجبن المطبوخ وجبن الفيتا على التوالي. تم فحص مائة عزلة مشتبه بها بيوكيميائيا. تم تشخيص 13 عزلة مصلية، منها 11 سلالة تم التأكد من كونها أنماط مصلية من STEC (O78:K80، O44:K74، O114:K90، O124:K63، O103:K- وO145:K-). تم فحص سبع سلالات تم الحصول عليها من الجبن القريش والتلاجة باستخدام تفاعل البلمرة المتسلسل لعوامل الضراوة مثل جينات سموم شيجا (stx1 وstx2) وجين الانتيمين (eaeA). أظهرت النتائج ان 3سلالات من أصل سبعة (42.9٪) لديها جينات stx1 و eaeA، 7/1 (14.3٪) تحمل جينات stx2 و eaeA، 7/1 (14.3٪) تحمل جينات stx1 و stx2 و eaeA بينما 7/2 (28.6٪) تحمل فقط الجين eaeA. تعتبر سلالات STEC التي تحتوي على الجينات المسئولة عن انتاج سموم الشيجا مع جينات الالتصاق (eaeA) تشكل خطرًا كبيرًا للإصابة بالأمراض لذلكيجب اتباع الشروط والإجراءات الصحية الصارمة أثناء إنتاج الجبن الطري وتداوله وبيعه بالأسواق لتفادى إصابة المستهلك بالامراض الخطيرة التي يسببها STEC وغيرها من الأ مراض التي تنتقل عن طريق الغذاء الملوث.

**الكلمات الدالة:** الإيشريشيا كولاى ، سموم الشيجا ، جينات الضراوة ، الجبن الطرى.