Occurrence of multidrug resistant shiga-toxigenic *E. coli* in retailed cheese in Zagazig city, Egypt
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**ABSTRACT**

Cheese is regarded as an essential dairy product that cover part of the human needs with vitamins and minerals. However, cheese is also implicated in many food poisonings outbreaks worldwide. This study conducted to estimate coliforms and *E. coli*, and to investigate the occurrence of shigatoxigenic *E. coli* in four cheese types mostly consumed in Zagazig city, Egypt. In addition, detection of coding genes for shiga toxins (*stx1* and *stx2*) was further screened using PCR. Besides, antimicrobial susceptibility of the isolated *E. coli* was further examined. The obtained results revealed that Kariesh cheese had the highest coliforms and *E. coli* counts, followed by Rumy, Domiati, and Feta cheeses, respectively. The isolation rates of *E. coli* were 52%, 24%, 20%, and 8% in Kariesh, Rumy, Domiati and Feta cheeses, respectively. The identified *E. coli* serotypes were *E. coli* O55:H7, *E. coli* O86:H11, *E. coli* O111:H4, *E. coli* O127:H6, and *E. coli* O26:H11. The expression of shiga toxin-coding genes indicated that *E. coli* O55:H7 harbored only *stx1*, *E. coli* O111:H4, and *E. coli* O86:H11 harbored only *stx2*, *E. coli* O78:H-, and *E. coli* O26:H11 harbored both *stx1* and *stx2*; however, *E. coli* O127:H6 did not express any of the tested genes. Antimicrobial sensitivity testing revealed multidrug resistance profiles, particularly among *E. coli* O86:H11, *E. coli* O78:H-, and *E. coli* O26:H11. Therefore, strict hygienic measures should be adopted during all manufacture steps of these kinds of cheese.

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1. **INTRODUCTION**

Cheese is rich in the essential amino acids, trace elements and minerals such as calcium and magnesium, and vitamins (Gerosa and Skoet, 2013; Ma et al., 2020). In Egypt there are many cheese types that are made from either raw or pasteurized milk such as Kariesh, Feta, Domiati, and Rumy cheeses. Cheese consumption is associated with many food poisonings outbreaks worldwide. One of the major organisms responsible for such food poisoning outbreaks is *Escherichia coli* (*E. coli*) (Mc Sweeney, 2007).

*E. coli* is a normal inhabitant in the intestinal flora of the farm animals. Detection of *E. coli* in foods of animal origin indicates fecal contamination. At the same time, *E. coli* is associated with several cases of human hospitalization (Darwish et al., 2015). *E. coli* strains are broadly classified into enteroaggregative (*ETEC*), enteropathogenic (*EPEC*), enterohemorrhagic (*EHEC*), enteroinvasive (*EIEC*), enteroaggregative (*EAEC*), and shiga toxin-producing *E. coli* (*STEC*). This classification is dependent on their pathogenicity and virulence attributes (Rutter et al., 2006; Xia et al., 2010). There are more than 100 *E. coli* serotypes that are classified as STEC. Of these, *E. coli* O157 causes 50 to 90% of *E. coli*-related human food poisoning cases, with most of the remaining cases caused by O45, O26, O111, O103, O121 and O145 (Scallan et al., 2011).

The uncontrolled usage of the antimicrobials in the animal production units had resulted in the development of antimicrobial resistance among microorganisms making treatment of infectious diseases as a challenging problem (Darwish et al., 2013). The present study was conducted to estimate the coliforms and *E. coli* counts and to examine the isolation rates of different *E. coli* serotypes in four cheese types (Kariesh, Feta, Domiati, and Rumy) mostly consumed in Zagazig city, Egypt. In addition, detection of shiga toxin-coding genes (*stx1* and *stx2*) was done using PCR. Besides, antimicrobial sensitivity testing of the identified serotypes was further examined using the disk diffusion method.

2. **MATERIAL AND METHODS**

2.1. **Sample collection:**

One hundred cheese samples of Kariesh, Feta, Domiati, and Rumy, (n = 25/each cheese type) were collected from different
grocery stores and street vendors in Zagazig city, Egypt. The microbiological examination of cheese samples was done at Faculty of Veterinary Medicine, Zagazig University.

2.2. Sample preparation:
Twenty-five grams from each sample was blended aseptically in buffered peptone water 0.1% (225 ml) for 2 min at 2500 rpm to obtain a dilution of 10³, followed by making decimal serial dilutions (APHA, 2001).

2.3. Estimation of MPN of coliforms:
The three tubes method recommended by APHA (2001) for the estimation of MPN of coliforms was followed.

2.4. Estimation of MPN of E. coli:
Loopfuls from the positive tubes (with acid and gas production) were aseptically inoculated into previously warmed tubes (44.5°C) containing 7 ml of E. coli (EC) broth (Himedia, Mumbai) and then incubated at 44.5°C for 24-48 hrs (APHA, 2001). The positive tubes were used to estimate MPN of E. coli according to the recommended tables.

2.5. Isolation of Escherichia coli:
From each positive tube (acid and gas) of EC broth, a loopful was streaked onto Eosin Methylene blue (EMB) agar plates, followed by incubation at 37°C for 24 hrs. Typical colonies of E. coli appear greenish, metallic with dark purple center. E. coli identification was done according to the staining and biochemical tests (APHA, 2001).

2.6. Sero-diagnosis of E. coli:
The rapid diagnostic E. coli antisera sets purchased from Difco, Detroit, USA were used for the serological identification of the confirmed E. coli isolates (Kok et al., 1996).

2.7. DNA preparation:
DNA was extracted from the confirmed E. coli isolates using the method reported before (Darwish et al., 2015).

2.8. Detection of shiga toxin-coding genes in the identified isolates:
Further detection of the coding genes for shiga toxins (stx1 and stx2) in the identified E. coli isolates was carried out using multiplex PCR. The primer sequences and the amplified products sizes were presented in Table 1. The amplification was performed on a Thermal Cycler (Master cycler, Eppendorf, Germany). PCR assays were carried out using the method of Dhanashree and Mallya (2008) using AmpliTaq DNA polymerase kit (Perkinelmer). E. coli O157:H7 Sakai was used as a positive strain for stx1, and stx2. While E. coli K12DH5n was used as a negative control. Visualization of the DNA fragments was done using agarose gel electrophoresis 2% in 1x TBE buffer. A 100 bp plus DNA Ladder was used as the experimental marker.

2.9. Antimicrobial sensitivity testing of E. coli:
Antimicrobial sensitivity testing was conducted using the disk diffusion method (Wayne, 2013). The tested antimicrobials were ampicillin (AM) 10 µg, cephalothin (CN) 30 µg, chloramphenicol (C) 30 µg, ciprofloxacin (CP) 5 µg, enrofloxacin (En) 5 µg, erythromycin (E) 15 µg, gentamicin (G) 10 µg, kanamycin (K) 30 µg, nalidixic acid (NA) 30 µg, neomycin (N) 30 µg, oxacillin (OX) 1 µg, oxytetracycline (T) 30 µg, penicillin (P) 10 IU and trimethoprim/sulfamethoxazole (SXT) 25 µg.

2.10. Statistical analysis
Coliforms and E. coli counts were expressed as means ± SE for log 10 cfu/g. Statistical analysis was done using Tukey–Kramer HSD test where, p <0.05 indicated statistical differences (Gomez and Gomez, 1984).

3. RESULTS
Figure 1A showed that Karieish cheese had the highest coliforms counts (log 10 cfu/g) among the examined cheese samples with a value of 4.15 ± 0.15, followed by Rumy cheese (3.87 ± 0.17), Domiati cheese (3.45 ± 0.21), and Feta cheese (2.45 ± 0.11), respectively. E. coli counts (log 10 cfu/g) in the examined cheese samples were corresponding to the coliforms counts as Karieish cheese had significantly the highest value (3.85 ± 0.18), followed by Rumy cheese (3.17 ± 0.12), Domiati cheese (2.75 ± 0.19), and Feta cheese (2.11 ± 0.22), respectively (Fig. 1B). The prevalence rates of E. coli were 52%, 24%, 20%, and 8% in Karieish, Rumy, Domiati and Feta cheeses, respectively (Fig. 2). The isolated E. coli were further identified into six serotypes namely E. coli O55:H7, E. coli O111:H4, E. coli O127:H6, E. coli O86:H11, E. coli O78:H, and E. coli O26:H11. Karieish cheese had the highest contamination level with different E. coli serotypes, particularly with E. coli O78:H (20%), and E. coli O26:H11 (16%), while Feta cheese had the lowest contamination level (Fig. 3A, B).

![Image](image-url)

Figure 1. Coliforms and E. coli counts in the examined cheese samples. Values represent means ± SE (Log 10 cfu/g) of A) Coliforms B) E. coli counts in Karieish, Feta, Domiati, and Rumy cheeses. Columns with different letter are significantly different at p < 0.05.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Oligonucleotide sequence (5’ → 3’)</th>
<th>Product size (bp)</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>stx1 (F)</td>
<td>5’ ACATCTGATGAGCTCAAGGCG 3’</td>
<td>614</td>
<td>Dhanashree and Mallya (2008)</td>
</tr>
<tr>
<td>stx1 (R)</td>
<td>5’ CTGAACTCCCTGCAAATATG 3’</td>
<td>779</td>
<td></td>
</tr>
<tr>
<td>stx2 (F)</td>
<td>5’ CCAAGAACCCGCACGACGTTG 3’</td>
<td></td>
<td></td>
</tr>
<tr>
<td>stx2 (R)</td>
<td>5’ CTAATGACACATGAGCAGTACTTGC 3’</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 1. Oligonucleotides’ sequences of shiga toxin-coding genes
We further detected the expression of shiga toxin-coding genes among the identified E. coli serotypes. E. coli O55:H7 harbored only stx1, E. coli O111:H4, and E. coli O86:H11 harbored only stx2. E. coli O78:H-, and E. coli O26:H11 harbored both stx1, and stx2; however, E. coli O127:H6 did not express any of the tested genes (Fig. 4).

Antimicrobial sensitivity testing revealed drug resistance for more than one antimicrobial, particularly in E. coli O86:H11, E. coli O78:H-, and E. coli O26:H11 (Table 2).

![Figure 2](image2.png)

Figure 2. Prevalence of E. coli in examined cheese samples.

Prevalence rates (%) of E. coli in Kariesh, Feta, Domiati, and Rumy cheeses (n=25 each)

![Figure 3](image3.png)

Figure 3. Prevalence rates of different E. coli serotypes A) in examined cheese types; B) Number and percentages of each serotype

![Figure 4](image4.png)

Figure 4. DNA expression of shiga toxins coding genes (stx1 & stx2) in the identified E. coli serotypes isolated from examined retail cheese samples.

Table 2: Antimicrobial sensitivity testing of E. coli serotypes identified in the examined cheese samples.

<table>
<thead>
<tr>
<th>No.</th>
<th>AM</th>
<th>SXT</th>
<th>OX</th>
<th>K</th>
<th>G</th>
<th>C</th>
<th>T</th>
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<td>O55:H7</td>
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<tr>
<td>O26:H11</td>
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<td>O78:H-</td>
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<td>100</td>
<td>0</td>
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</tr>
</tbody>
</table>

No.: Number of isolates
%: Percentage of isolates
AM: Ampicillin; CN: Cephalothin; C: Chloramphenicol; CP: Ciprofloxacin; En: Enrofloxacin; E: Erythromycin; G: Gentamicin; K: Kanamycin; NA: nalidixic acid; N: Neomycin; OX: Oxacillin; T: Oxytetracycline; P: Penicillin; SXT: trimethoprim/sulfamethoxazole

4. DISCUSSION

In the present study, Kariesh cheese had the highest coliforms counts as well as, E. coli counts among the examined cheese samples followed by Rumy, Domiati then Feta cheeses, respectively. Such high values of coliforms and E. coli counts reflect improper hygienic measures adopted during cheese preparation, storage, or distribution, particularly in the case of Kariesh cheese (Mossel et al., 2019). Similarly, unsatisfactory hygienic measures were reported for the retailed fresh cheese in Mexico (de la Rosa et al., 2019). Furthermore, unsatisfactory hygienic measures were identified in Canada, between October 2002 and February 2003 (Honish et al., 2005). Besides, E. coli O104:H4 was responsible for an outbreak occurred in Germany in May 2011 infecting more than 3000 peoples and left 50 deaths (Frank et al., 2011). In addition, another 19 persons were infected with the shiga toxin producing E. coli O121 in six of United States (CDC 2014). In the present study, the highest prevalence rate of E. coli was in Kariesh cheese followed by Rumy, Domiati and Feta cheeses, respectively. In agreement with the obtained results in the present study, Hassan and Elmalt (2008) isolated toxigenic E. coli from retailed Kariesh cheese in Qena city at 47.8%. Besides, Oombarak et al. (2016) isolated E. coli of enteropathogenic and enterohemorrhagic types at 74.5% from Kariesh cheese, and 21.7% from Ras cheese retailed in Egypt. Furthermore, Hussein et al. (2019) isolated E. coli at 16%, and 5.3% from Kariesh cheese and Ras cheese, respectively, sold in Menoufia Governorate.

E. coli O55:H7, E. coli O111:H4, E. coli O127:H6, E. coli O86:H11, E. coli O78:H-, and E. coli O26:H11 were further identified as the prevalent serotypes in the current investigation. Kariesh cheese had the highest contamination level with different E. coli serotypes, particularly with E. coli O78:H- and E. coli O26:H11, while Feta cheese had the lowest contamination level. Similarly, de Campos et al. (2018) could identify E. coli O127, O73:H12, and O64474:H8 from Minas cheese in Brazil. Furthermore, Hussein et al. (2019) identified eight E. coli serotypes from Kariesh and Ras cheeses, namely, E. coli O26: H11, O91: H21, O111: H2, O103: H2, O125: H21, O171: H2, O86:H, and O119: H6.
The expression of shiga toxin-coding genes among the identified _E. coli_ serotypes were detected. The obtained results revealed that _E. coli_ O55:H7 harbored only stx1, _E. coli_ O111:H4, and _E. coli_ O86:H11 harbored only stx2, _E. coli_ O78:H1, and _E. coli_ O26:H1 harbored both stx1, and stx2; however, _E. coli_ O127:H6 did not express any of the tested genes. _E. coli_ of non-O157 serogroups such as O26, O103, O111 were reported to be the most substantial food poisoning pathogen groups, especially O26 that able to cause wide range of illness in human (Dambrosio et al., 2007). Similarly, _E. coli_ expressing shiga toxin coding genes were isolated from a Spanish raw ewe’s milk cheese (Caro et al., 2007). In addition, Elhadidy and Mohammed (2013) isolated shiga toxin-producing _E. coli_ including serotypes _O22-H2, O26-H11, O86-H21, O103-H2, O113-H21 and O146-H21_ from Kariesh and Domiati cheese retailed in Egypt. Hussein et al. (2019) could also identify eight _E. coli_ serotypes from Kariesh and Ras cheeses producing shiga toxins (stx1, and stx2). STEC is implicated in many cases of hemorrhagic colitis, hemolytic uremic syndrome, and thrombotic thrombocytopenic purpura (Karch et al., 2005). The uncontrolled usage of antimicrobials in the livestock production is the major cause for the development of drug-resistance pathogens, which is regarded as a major health concern. In the current study, the multidrug resistance profiles particularly among _E. coli_ O86:H11, _E. coli_ O78:H-, and _E. coli_ O26:H11 were prominent. For instance, 50% or more of the identified _E. coli_ O26:H11 showed resistance to AM, P, NA, and OX. While 50% or more of _E. coli_ O86:H11 showed resistance to AM, G, NA, P, OX, and SXT. Isolation of multidrug resistant _E. coli_ from cheese was reported in studies conducted in Romania (Tabaran et al., 2017), Brazil (de Campos et al., 2018), and Egypt (Ombarak et al., 2018). Therefore, rational use of antimicrobials in the animal farms and livestock production is highly recommended. In conclusion, the current study revealed isolation and identification of multidrug resistant and shiga toxin-producing _E. coli_ from Kariesh, Domiati, Rumy and Feta cheese retailed in Zagazig city, Egypt. Therefore, strict observation of hygienic measures should be adopted during all manufacture steps of these kinds of cheese. In addition, continuous monitoring studies for the prevalence of STEC in other dairy products are highly recommended. 

**REFERENCES**

3. Centers for Disease Control and Prevention (CDC), 2014b. Multistate Outbreak of Shiga toxin-producing _Escherichia coli_ O121 Infections linked to raw Clover Sprouts (Final Update).