Occurrence of Virulent and Antibiotic-resistant Enteropathogenic and Shiga toxin-producing *Escherichia coli* in some Milk Products Sold in Assiut City, Egypt

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**ABSTRACT**

This study was undertaken to detect the enteropathogenic and shiga toxin-producing *Escherichia coli* (EPEC and STEC) in 120 milk products samples (soft cheese, hard cheese, yoghurt and ice cream). All samples were submitted for bacteriological examination, serological and molecular identification of virulent and antibiotic resistant genes using *eaeA*, *hlyA*, *blaTEM*, *blaCTX-M*, *blaOXA* and *blaSHV* primers. The bacteriological examination revealed that the incidence of occurrence of EPEC was 3.33% in ice cream samples, while it could not isolated from other types of milk products. In addition, STEC failed to detect in all examined milk products. The isolated EPEC strain following *E. coli* O18 serotyping. Moreover, the molecular identification of the isolated strain revealed that the strain contains *eaeA*, *blaTEM* and *blaCTX-M* genes.

**Keywords:**

EPEC, STEC, *E. coli* O18, milk products, *eaeA*, β-lactamase genes

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**Introduction**

Milk products are considered the most popular Egyptian food and play a prominent role in the Egyptian diet. The manufacture of these products is based on traditional method without any regard to the quality of raw material used and/or the hygienic quality of the products. Beside their manufacture, the handling techniques in Egyptian markets are still primitive and unhygienic.

Many enteropathogenic microorganisms have been found in milk products specially that not subject to efficient heat treatment. They are frequently associated with several outbreak (Almeida Filho and Nader Filho, 2002). One of these bacteria is *Escherichia coli*, which is normally inhabit in intestine of animal and human (Wetzel, 2005). Pathogenic strain of *E. coli* can be divided according to the mechanism of action into diarhoeagenic *E. coli* and extra intestinal *E. coli* (Croxall et al., 2011) based on the site of infection. *E. coli* strain harbor various virulence factors including enteropathogenic *E. coli*, enterotoxigenic *E. coli* (ETEC), enterohemorrhagic/ Shiga toxin-producing *E. coli* (STEC), enterogaegregative *E. coli* (EAEC) and diffusely adherent *E. coli* (DAEC) (Kaper et al., 2004) which lead to gastrointestinal tract disease, including watery or bloody diarrhea and might develop life-threatening disease. While, extra intestinal *E. coli* infection causes many diseases as uropathogenic *E. coli* (UPEC) causing urinary tract infection, *E. coli* strains that cause septic anemia, and neonatal meningitis associated *E. coli* (MNEC) (Bekal et al., 2003).

Many virulence factors responsible for previous diseases as two phage encoded cytotoxins (stx1 and stx2) (Kawano et al., 2012), intimin (*eaeA*) and hemolysin (*hlyA*) (Slanec et al., 2009). The most prevalent serotypes with the enteropathogenic *E. coli* (EPEC) are O18, O20, O25, O26, O44, O55, O86, O91, O111, O114, O119, O125 ac, O126, O127, O128, O14 and O158 (Nataro and Kaper, 1998) these serotypes linked epidiemologically to infantile diarrhea. While, the most prevalent serotypes associated with (STEC) human infections are O157, O111, O26, O103, O113, O91, O118, O121, O145, O128 and O146 (Rahal et al., 2015).

The treatment of gastroenteritis caused by *E. coli* requires antimicrobial therapy. However, currently there has been a rise in the occurrence of antibiotic-resistant *E. coli* due to the presence of resistance genes in *E. coli*. These genes responsible for production of hydrolysis enzymes, which lead to destruction

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of the beta-lactamase ring of the antibiotics. These enzymes known as extended spectrum β-lactamases (ESBLs) such as TEM, CTX, OXA and SHV enzymes (Bush and Jacoby, 2010).

Considering the above facts the present study was designed to determine the occurrence of virulence and antibiotic-resistant genes profile of enteropathogenic E. coli isolated from some milk products sold in Assiut City, Egypt.

Materials and methods

Sample and study area

A total of 120 random samples of some milk products including: soft cheeses (Kareish and Damietta), hard cheeses (Cheddar and Ras), yoghurt and ice cream (30 for each) were been collected from different places in Assiut City in clean, dry and sterile containers, while ice cream samples were taken in the ice boxes. These samples were transferred to the laboratory as soon as possible to be examined.

Preparation of samples

Twenty five grams from each sample were thoroughly mixed before being examined according to A.P.H.A. (2004).

Isolation of EPEC and STEC

Enrichment procedure (Samadpour et al., 1990)

A tube of modified vancomycin-tryptase soya broth (mvTSB) was inoculated with 1 ml prepared sample. Mixed well and incubated at 37 °C for 24.0 ± 2.0 hrs.

Plating on selective agar media (A.P.H.A., 2004)

A loopful of incubated broth was subcultured into the surface of Sorbitol MacConkey (SMAC) agar followed by incubation at 37°C for 24 hrs.

Identification of EPEC

Biochemical tests

Triple Sugar Iron (TSI), Indol test, Methyl Red Test, Voges Proskaur test and Citrate utilization test were used as biochemical tests to identify the isolated strains (A.P.H.A., 2004).

Serological tests

The identified E. coli isolate was serotyped by slide agglutination test in the serological laboratory of Animal Health Research Institute (Cairo, Egypt) using standard polyvalent and monovalent EC antisera according to Hamed et al. (2017).

Polymerase chain reaction (PCR)

DNA extraction

The suspected bacterial colony was subcultured onto TSB and incubated overnight at 37°C for DNA extraction using Qia-gen DNA blood Mini kit (Cat.No. 51104, Hilden, Germany) according to manufacturer instruction. The extracted DNA was stored at -20°C.

DNA amplification was performed in final volume 25 μl, which contained the following, 12.5 μl of 2X PCR master mix (Green Master, Promega, USA), 150 ng of the DNA template, 0.5 μM of each primer (Table 1) and up to 25 μl Nuclease free water, the mixture was mixed in a PCR tube. The virulent factors genes amplification was performed in a programmable gradient thermal cycler (Veriti Applied Biosystem, USA) at 94°C for five min, followed by 35 cycles were run under the following conditions; denaturation at 94°C for 50 sec, annealing at 63°C for 50 sec and extension at 72°C for 1 min. At the end, the preparations were kept for 10 min at 72°C as final extension. While, the thermal profile of antibiotic-resistant genes amplification was at 95°C initial denaturation step for 5 min followed by 35 cycles of 95°C for 40 sec, 51°C for 40 sec, and 72°C for 90 sec. The final cycle was followed by 72°C incubation for 10 min.

Gel Electrophoresis

PCR products were electrophoresed in 1% agarose gel (GX 040.90, Gen Agarose, L.E., Standard DNA /RNA agarose, Molecular Biology Grade, Inno–Train Diagnostic, D–61476, Kronberg/Taunus) Containing Ethidium bromide as 1µl /ml electrophoresis buffer at 100 volts for 60 min. Using 100 bp DNA–ladder in (SCiE–PLAS, HU 10, 5636, UK). The result obtained through High performance ultraviolet Transilluminator,

Table 1. Primers used to amplify fragments of genes responsible for expression of different virulence factors and antibiotic-resistant genes for E. coli.

<table>
<thead>
<tr>
<th>Primer Name</th>
<th>Sequence (5’-3’)</th>
<th>Target gene</th>
<th>PCR product (bp)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>AE22</td>
<td>ATTACCATCACAACAGCAGCT</td>
<td>aaeA</td>
<td>397</td>
<td>Sarinehmetoglu et al. (2009)</td>
</tr>
<tr>
<td>AE20-2</td>
<td>ACAGCCTGGTTGGTGAACCT</td>
<td>bly</td>
<td>166</td>
<td></td>
</tr>
<tr>
<td>MFS1-F</td>
<td>ACGTGAGTTATCTGGAAT</td>
<td>blaTEM</td>
<td>800</td>
<td>Oguta et al. (2015)</td>
</tr>
<tr>
<td>MFS1-R</td>
<td>CTCCACGCTA1ACCACATA1AT</td>
<td>blactX</td>
<td>655</td>
<td></td>
</tr>
<tr>
<td>BiaTEM-F</td>
<td>CGTTTCGCTG7CGCCCTTAATC</td>
<td>bIaoxA</td>
<td>564</td>
<td>Perez et al. (2007)</td>
</tr>
<tr>
<td>BiaTEM-R</td>
<td>CGTTTCGCTGATTTGCTGAC</td>
<td>blashF</td>
<td>713</td>
<td></td>
</tr>
<tr>
<td>BiaCTX-M1-F</td>
<td>GGCGGAGGATCTTATCTTCAAG</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BiaCTX-M1-R</td>
<td>CGTTTTATCCCAAAACCAC</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BiaOXF-F</td>
<td>GCCACAGATTCAACTTTTCAAG</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BiaOXF-R</td>
<td>GACCCCAAGTTTTTCTCTGTAAGTG</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BiaSHF-F</td>
<td>AGGCCGATTGAGCAAAATTAACAC</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BiaSHF-R</td>
<td>ATCCCGACAGATAATACACCAC</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
The image of the PCR products containing the positive DNA sequence of the positive genes were documented using DOC–It ® LS, Image acquisition–software, (Biodoc Analyzer, Biometra, Germany).

Results

Results of this study revealed that EPEC could be detected only on one ice cream sample in percent 3.33%, while it could not be isolated from the soft cheese, hard cheese and yoghurt samples. Moreover, STEC didn’t detect in the examined milk products (Table 2).

In addition, this study carried out the serotyping of isolated EPEC. The strain following E. coli O18 group with polyvalent 3, also, the strain contain eaeA, blatEM and blCTX_M1 genes, on the other side, the strain didn’t carry hylA, bladEA and blasNV as described in Table 3 and Fig. 1.

Discussion

Milk products have been associated with health benefits for many years containing bioactive peptide, probiotic bacteria, antioxidant, vitamins and highly absorbable calcium (Bhat and Bhat, 2011). However, the method of production, transportation, handling and sale of milk and milk products are entirely unhygienic which lead to contamination by food poisoning bacteria as E. coli.

The results of milk product samples revealed that the ice cream samples were contaminated with EPEC in percentage of 3.33. While, the others products (soft cheese, hard cheese and yoghurt samples) didn’t contaminated by E. coli. By the serotyping the isolated strain was E. coli O18. The ice cream contaminated by E. coli O18 may be attributed to contamination of milk used in the manufacturing or due to post pasteurization and post processing contamination. On the other side, it didn’t detect in the soft cheese, hard cheese and yoghurt samples due to the low pH of the previous products, which inhibit the growth of these bacteria. These result agree with Elbagory et al. (2015) result, they didn’t isolate E. coli O18 from Kareish and Damietta cheese, while they isolate it from raw milk samples. Moreover, STEC as E. coli O157 failed to detect in the examined milk products. These results agree with that carried out by Ibrahim and Sobeih (2006) and Nazem et al. (2016), they also failed to detect E. coli O157:H7 from cheese samples.

E. coli O18 implicated in many outbreak as an outbreak of bloody diarrhea occurred in the neonatal nursery ward in Argentina, in 1996 (Chinen et al., 2002). In addition, this strain is involved in a large range of extra intestinal infections in human as neonatal meningitis, septicemia, pyelonephritis and pneumonia (Russo and Johnson, 2000; Johnson and Russo, 2002; Mokady et al., 2005; Ron, 2006), as well as, in animals such as mastitis in dairy animals (Lamey et al., 2013).

Defining the virulence factors and mechanism of E. coli O18 pathogenesis has been on many literatures. The production of eaeA and hylA are considered essential but not solely responsible for disease. In our study, the isolate was carried eaeA gene, while, the isolated strain wasn’t contain hylA genes. The most important protein facilitate the pathogenicity of EPEC is intimin. Intimin is outer membrane protein encoded by eaeA gene which help the E. coli to intimate attachment to intestinal epithelial cells. The affected epithelial cells include phosphorylation, calcium influx and the rearrangement of cytoskeletal components under the adherent EPEC. So, it lead to reduce absorption capacity of the brush border and a stimulation of intestinal secretion (Wilshaw et al., 2000).

The first pathotype of E. coli was the EPEC. This type was isolated from large outbreaks of infant in UK, in 1945 (Kaper et al., 2004). The most lineament of EPEC infections is the attaching-effacing histopathology, which can be detected by intestinal biopsy specimens from patients or infected animals. In addition, EPEC infection is primarily a disease of infants

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Table 2. Incidence of EPEC and STEC in the examined milk products samples

<table>
<thead>
<tr>
<th>Examined samples</th>
<th>No. of examined samples</th>
<th>EPEC Positive samples</th>
<th>STEC Positive samples</th>
</tr>
</thead>
<tbody>
<tr>
<td>Soft cheese</td>
<td>30</td>
<td>-</td>
<td>0</td>
</tr>
<tr>
<td>Hard cheese</td>
<td>30</td>
<td>-</td>
<td>0</td>
</tr>
<tr>
<td>Yoghurt</td>
<td>30</td>
<td>-</td>
<td>0</td>
</tr>
<tr>
<td>Ice cream</td>
<td>30</td>
<td>1</td>
<td>3.33</td>
</tr>
</tbody>
</table>

Table 3. Characterization of virulence factors and antibiotic-resistant of E. coli O18 by PCR.

<table>
<thead>
<tr>
<th>Target Gene</th>
<th>Result</th>
<th>Target Gene</th>
<th>Result</th>
</tr>
</thead>
<tbody>
<tr>
<td>eaeA</td>
<td>Positive</td>
<td>hyl</td>
<td>Negative</td>
</tr>
<tr>
<td>blatEM</td>
<td></td>
<td>bladEA</td>
<td></td>
</tr>
<tr>
<td>blCTX_M1</td>
<td></td>
<td>blasNV</td>
<td></td>
</tr>
</tbody>
</table>

Fig. 1. PCR products of amplified of virulent and antibiotic resistant genes visualized on agarose gel electrophoresis. Lane (M) DNA ladder 100 bp, Lane (1) positive for gene eaeA at 397 bp, Lane (2) positive for gene blatEM at 800 bp, Lane (3) positive for gene blCTX_M1 at 655 bp.
younger than 2 years (Nataro and Kaper, 1998). Beside the watery diarrhea, which is hallmark sign of EPEC, the vomiting and low-grade fever are common symptoms of it. Some studies in Brazil, Mexico, and South Africa have shown that 30-40% of infant diarrhea can caused by EPEC (Robins-Browne et al., 1980; Cravioto et al., 1990; Gomes et al., 1991).

**E. coli** has become the most important member for infection and colonization of food animals since the early 2000s (Aidara-Kane et al., 2013; Lahlou et al., 2014; Rao et al., 2014; Su et al., 2014). In this study, the isolated *E. coli* O18 strain contained two genes responsible for ESBL as *bla*TEM and *bla*CTX-M-1, while the strain didn’t have *bla*OXA and *bla*SHV genes. These result similar to the result obtained by Gundogan and Avci (2013), they detected only one strain of ESBL producing *E. coli* isolated from ice cream milk samples sold in Turkey. Many previous studies recorded the ESBL producing *E. coli* in raw milk and researchers concluded that the unclean hand worker, unhygienic conditions of milking, unclean water supplies used in washing the utensils, all the previous factors play an important role in contamination of the milk and milk products with antibiotic-resistant *E. coli*. In addition, the intensive use of antibiotics for animal production lead to increase the incidence of the antimicrobial resistance bacteria in milk and milk products (Tekiner and Ozpinar, 2016; Badri et al., 2018 and Ombarak et al., 2018).

**Conclusion**

The results obtained from this study concluded that the milk products sold in the Assiut markets have EPEC contamination. Thus, the results of the study warn the need more strict preventive measure. So, certain recommendations should be followed as implementation of hygienic measures in dairy farms and dairy plants. Also, the present study suggest investigating the HACCP application to avoid milk products contamination by EPEC.

**Acknowledgement**

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**References**


