Phenotypic and genotypic aspects of sorbitol-negative Escherichia coli isolated from cattle

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Phenotypic and genotypic aspects of sorbitol-negative *Escherichia coli* isolated from cattle

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**ABSTRACT:** *Escherichia coli* especially sorbitol-negative serogroups such as O157 that produce Shiga toxin (STEC) are involved in food poisoning with severity ranging from individual cases to outbreaks that threaten human and animal health. This study aimed to evaluate phenotypic and genotypic aspects of sorbitol-negative *E. coli* isolated from different sources. A total of 420 samples were collected from fecal swabs and raw milk of cattle from various locations in Kafr Elsheikh governorate, Egypt. The prevalence of *E. coli* isolates was 9.3% (39/420) as was determined by bacterial culturing. The phenotype of these isolates was serologically and molecularly determined, and the results showed an overall prevalence of *E. coli* O157 of 23% (9/39) with a high incidence in fecal swab samples. The multidrug resistance (MDR) phenotype as detected by antibiotic sensitivity test and confirmed by PCR revealed resistance to amoxicillin, clavulanic acid, vancomycin, ampicillin, and sulbactam with the detection of *blaTEM* and *blaSHV* MDR genes in 9 and 2 isolates, respectively. Virulence genes (*stx1, stx2, eaeA*) were also detected in 1, 9, 6 isolates, respectively. With these results, we could conclude that *E. coli* O157 was identified in not only cattle fecal swab samples but also in their milk and subsequently this could threaten animal and human health.

**Keywords:** *E coli* O157; virulence factors; antibiotic resistance; STEC; milk

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INTRODUCTION

Escherichia coli is a member of the family Enterobacteriaceae, facultatively anaerobic, Gram-negative short rods and counted a common occupant of the stomach of warm-blooded animals, together with man, but similarly be in the water, soil, or other environments due to fecal contamination (Bujňáková et al., 2021). Based on virulence, the enteric E. coli is categorized into enterotoxigenic (ETEC), enteropathogenic (EPEC), enteroinvasive (EIEC), verotoxigenic (VTEC), enterohemorrhagic [ (EHEC, which is a subgroup of Shiga toxin-producing E. coli (STEC)], diffusely adherent (DAEC), enteropathogenic (EPEC), enteroaggregative (EAEC), and necrotic (NTEC) (Hammerum and Heuer, 2009). Among the different serogroups, E. coli O157 is a foodborne pathogen that can cause hemorrhagic colitis (HC), hemolytic-uremic syndrome (HUS), watery and/or bloody diarrhea, and thrombotic thrombocytopenic purpura (TPP) in infected human (Bandyopadhyay et al., 2011, Shahreza et al., 2017). Food animals such as cattle are the main animal reservoir of E. coli O157 (Ferens and Hovde, 2011, Yan et al., 2011). Humans can be infected by the consumption of either contaminated animal milk (Lika et al., 2021) or water, and food contaminated with food animal and all companion animal fecal matter (Amézquita-López et al., 2016, Puvača and de Llanos Frutos, 2021). Moreover, STEC was considered as one of the normal flora inhabitants of the animal intestine (Hammerum and Heuer, 2009, Ibekwe et al., 2011). This increases the prevalence of human infection.

E. coli O157 pathogenicity depends mainly on virulence factors such as Shiga toxin and intimin (Ojo et al., 2010, Shahreza et al., 2017). Intimin that is encoded by the eaeA gene is essential for attaching, while Shiga toxins are responsible for binding to the glycolipid globotriaosylceramide (Gb3) on the target cell surface preventing protein synthesis and leading to the death of infected cells (Bandyopadhyay et al., 2011, Amézquita-López et al., 2016, Mir et al., 2016, Shahreza et al., 2017).

This study was conducted to investigate phenotypic and genotypic aspects of sorbitol negative E. coli isolates collected from cattle fecal swabs and milk samples.

MATERIALS AND METHODS

Isolation and identification of E. coli

A total of 420 samples were collected from diarrheic cattle’s fecal swabs and raw milk in the Kafrel-Sheikh governorate. Fecal swab (230) and milk (190) samples were collected from private veterinary clinics. Collected samples were first enriched in tryptic soy broth for 24 h, then 100 μl were cultured onto Eosin Methylene Blue agar (Difco) to presumably detect E. coli (green-metallic colonies), and finally, these colonies were plated onto Sorbitol MacConkey agar (Difco) to determine sorbitol non-fermenting bacteria (colorless colonies, n = 39). The latter were plated on Tryptic Soy agar and were identified morphologically by Gram staining and biochemically by sugar fermentation, indole production, methyl-red, and Voges Proskauer tests (Quinn et al., 2002).

Serological identification of E. coli O157

E. coli isolates were serotyped in Animal Health Research Institute, Dokki, Giza using standard monovalent E. coli O157 antisera as previously described (Edwards and Ewing, 1972).

Antimicrobial drug sensitivity test

The sensitivity test was performed using the disk diffusion method which was performed using Muller-Hinton agar and 16 antibiotic disks (listed in Table 3) as previously described (Jorgensen JH et al., 1997). The antibiotic sensitivity of isolates was measured based on the diameters of growth inhibition zones. The results were interpreted based on the Clinical and Laboratory Standards Institute Guidelines (Clinical and Institute, 2011).

Polymerase chain reaction (PCR)

Genomic DNA was isolated from E. coli isolates using a commercially available kit (QIAamp, Qiagen, GmbH, Germany) following the manufacturer’s instructions and as previously detailed (Allam et al., 2019). Polymerase chain reaction (PCR) was used to detect virulence (stx1, stx2, eaeA) and drug resistance (blaTEM and blaSHV) genes of E. coli using specific primers (Table 1). A PCR reaction mixture (30 μl) was set and included 5 μl genomic DNA, 15μl 2X Master mix (Emerald Amp GT, Takara, Japan), 1 μl from each primer (20 pmol), and 8 μl nuclelease-free water. The PCR thermal cycler (Applied biosystem 2720) was set with the following thermal conditions: 94°C/5 min (initial denaturation), followed by 35 cycles of 94°C/30 sec (denaturation), 58°C for stx1, stx2, eaeA, 51°C for eaeA, 54°C for blaTEM and blaSHV (annealing temperature for 40 sec), 72°C/45 sec (extension), followed by 72°C/10 min (final extension). Agarose gel
(1.5%) electrophoresis (Applichem, GmbH, Germany) with a DNA 100 bp marker (Qiagen, GmbH, Germany) was applied to determine PCR product size. The gel was photographed by a gel documentation system (Alpha Innotech, Biometra).

RESULTS AND DISCUSSION

Prevalence of E. coli O157

Morphological and biochemical identification revealed the presence of a total of 39 positive E. coli isolates recovered from 420 samples with an overall prevalence of 9.3% (Table 2). The prevalence of the positive isolates distributed based on their source was 12.2% (28/230) from diarrheic cattle’s fecal swabs and 5.8% (11/190) from raw milk. Based on serological identification, a total of 9 positive E. coli O157 isolates recovered from the positive 39 E. coli isolates with a prevalence of 23.1% (Table 2). The distribution of E. coli O157 based on the source of the samples was 28.6% (8/28) from diarrheic cattle’s fecal swabs and 9.1% (1/111) from raw milk. This infers that the recovery rate of E. coli O157 isolates varies according to the sample source. We found a higher incidence in diarrheic cattle’s fecal swabs than raw milk. E. coli O157 recovery rate was close to that obtained by Hussein and Bollinger (2005) but higher than that reported by Cernicchiaro et al. (2012) who reported a recovery rate of 27.8% and 19%, respectively in cattle fecal matter. In contrast, Selim et al. (2013) did not find E. coli O157 in cattle fecal samples but rather recovered E. coli O26. Regarding the raw milk samples, our results agree with Mohamed et al. (2003) who found an E. coli O157 recovery rate of 7.1%. However, Abdul-Raouf et al. (1996) reported a lower prevalence rate of 6% in raw milk.

Antimicrobial resistance profile and antibiotic resistance genes of E. coli O157

The massive use of antimicrobial drugs in animal farms is one of the most causative factors responsible for the development of microorganisms that resist antibiotics (Darwish et al., 2013). The antibiotic resistance profile of the isolated E. coli O157 serogroups was presented in Table 3. E. coli O157 isolates were 100% sensitive to Tetracycline, Ciprofloxacin, Ofloxacin, Norfloxacin, and Azithromycin. E. coli O157 isolates showed high to moderate sensitivity to Cloxacillin, Ampicillin, Cefoxitin, Sulfamethoxazole, Doxycycline, Cefoperazone, Erythromycin, and Amikacin. However, E. coli O157 isolates showed 100% resistance against Amoxicillin + Clavulanic acid, Ampicillin + Sulbactam, and Vancomycin. These results are consistent with those obtained by Selim et al. (2013) who found high resistance of E. coli O157 isolated from stool to Amoxicillin + Clavulanic acid, Ampicillin + Sulbactam, and Vancomycin. The presence of antibiotic-resistant strains of E. coli signifies the main health threat and indicates that Amoxicillin

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward</th>
<th>Reverse</th>
<th>Size (bp)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stx1</td>
<td>ACACCTGGATGATC</td>
<td>CTGAATCCCCCTC</td>
<td>614</td>
<td>(Dipineto et al., 2014)</td>
</tr>
<tr>
<td>Stx2</td>
<td>CCATGACAACGGA</td>
<td>CCGTCAACTGAGC</td>
<td>779</td>
<td></td>
</tr>
<tr>
<td>eaeA</td>
<td>ATGCTTAGTCTGG</td>
<td>GCCCTCATCTTCT</td>
<td>248</td>
<td>(Bisi-Johnson et al., 2011)</td>
</tr>
<tr>
<td>blaTEM</td>
<td>ATCGAATAAAC</td>
<td>CCCGAAGAAGGT</td>
<td>516</td>
<td>(Colom et al., 2003)</td>
</tr>
<tr>
<td>blaSHV</td>
<td>AGGATGACTGCC</td>
<td>ATTTGCTGATCCG</td>
<td>392</td>
<td></td>
</tr>
</tbody>
</table>

### Table 2. Prevalence of E. coli in the examined samples

<table>
<thead>
<tr>
<th>Sample</th>
<th>No of samples</th>
<th>No. of E. coli positive samples</th>
<th>Prevalence (%)</th>
<th>No. of E. coli O157 positive samples</th>
<th>Prevalence (%)**</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fecal swab</td>
<td>230</td>
<td>28</td>
<td>12.2</td>
<td>8</td>
<td>28.6</td>
</tr>
<tr>
<td>Milk</td>
<td>190</td>
<td>11</td>
<td>5.8</td>
<td>1</td>
<td>9.1</td>
</tr>
<tr>
<td>Total</td>
<td>420</td>
<td>39</td>
<td>9.3</td>
<td>9</td>
<td>23.1</td>
</tr>
</tbody>
</table>

%*: No of E. coli positive samples / No of examined samples.
%**: No of E. coli O157 positive samples / No of E. coli positive samples.
+ Clavulanic acid, Ampicillin + Sulbactam, and Vancomycin could have limited use. Having in mind high antimicrobial resistance towards mentioned antibiotics, usage of natural alternatives or essential oils qualitative and quantitative mixtures could be promising solution (Mileva et al., 2020, Khan et al., 2022).

Table 3. Results of antimicrobial sensitivity test for E. coli O157.

<table>
<thead>
<tr>
<th>Antimicrobial agent</th>
<th>Resistant</th>
<th>Sensitive</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No.</td>
<td>%</td>
</tr>
<tr>
<td>Cloxacillin</td>
<td>2</td>
<td>22.2</td>
</tr>
<tr>
<td>Ampicillin</td>
<td>3</td>
<td>33.3</td>
</tr>
<tr>
<td>Cefoxitin</td>
<td>1</td>
<td>11.1</td>
</tr>
<tr>
<td>Cefotaxime</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Sulfamethoxazole</td>
<td>2</td>
<td>22.2</td>
</tr>
<tr>
<td>Amoxicillin + Clavulanic acid</td>
<td>9</td>
<td>100</td>
</tr>
<tr>
<td>Ampicillin + Sulbactam</td>
<td>9</td>
<td>100</td>
</tr>
<tr>
<td>Tetracycline</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Doxycycline</td>
<td>2</td>
<td>22.2</td>
</tr>
<tr>
<td>Cefoperazone</td>
<td>1</td>
<td>11.1</td>
</tr>
<tr>
<td>Ciprofloxacin</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Ofloxacin</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Norfloxacin</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Erythromycin</td>
<td>1</td>
<td>11.1</td>
</tr>
<tr>
<td>Azithromycin</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Amikacin</td>
<td>2</td>
<td>22.2</td>
</tr>
<tr>
<td>Vancomycin</td>
<td>9</td>
<td>100</td>
</tr>
</tbody>
</table>

Beta-lactams are extensively utilized in the treatment of diseases caused by E. coli in animals. However, their massive use could result in the development of Beta-lactams resistant strains of E. coli. Transmission of antibiotic resistance genes, such as TEM and SHV β-lactamase-encoding genes (blaTEM and blaSHV) to humans should be considered when farm animals are infected with MDR bacterial strains (Manges et al., 2007). In the present study, we detected blaTEM in all E. coli O157 isolates (n = 9) with a prevalence rate of 100% (Figs. 1, 2). However, BlaSHV was detected only in 2 isolates with a prevalence rate of 22.2%. Based on the source of the sample, the fecal samples showed a higher incidence of 100% (8/8) for blaTEMand 100% (1/1) for BlaSHV than milk samples which showed 25% (2/8) for blaTEM while no BlaSHV was detected. This implies that the results of the sensitivity test agree with that of PCR and confirm the presence of antibiotic resistance genes. In support, other studies also detected blaTEM and BlaSHV in animal fecal samples but with different prevalence of 89% and 42.9%, respectively (Rocha-Gracia et al., 2014). E. coli O157 expressing blaTEM and BlaSHV constitute multidrug resistance (Hosu et al., 2021).

With these findings, we could conclude that many diarrheic animals harbor β-lactam-resistant E. coli which threatens the health of humans and animals.

Virulence genes

Shiga toxins and Intimin play a crucial role in the pathogenicity of STEC (Ojo et al., 2010). Shiga toxins participate in the attachment of bacterial holotoxin to the cell membrane of the host cells and inhibit protein synthesis leading to the destruction of affected cells (Newton et al., 2009). The Intimin, which is encoded by the eaeA gene, participates in the development of
Attaching/Effacing (A/E) lesion which destroys the microvilli and helps bacterial colonization into the lining epithelia of the intestine leading to the hemolytic uremic syndrome in humans (Woodward et al., 2003).

In the present study, PCR was used to detect stx1, stx2, and eaeA virulence genes in the 9 pathogenic E. coli O157 isolates (Figs. 3-5). The incidence rates were 11.1% (1/9) for the stx1 gene, 100% (9/9) for the stx2 gene, and 66.6% (6/9) for the eaeA gene. Both stx2 and eaeA virulent genes were detected in fecal samples (8/8, 5/8) and raw milk (1/1), respectively. However, Stx1 was only detected in fecal samples.

Our findings regarding the prevalence of stx1 and stx2 genes in fecal samples of cattle are compatible with those obtained by Barkocy-Gallagher et al. (2001) and Khanjar and Alwan (2014). Similarly, stx1 and stx2 genes were detected in 38% of E. coli isolates of raw milk and beef meat contaminated with feces of animals (Shahzad et al., 2013). Moreover, Elder et al. (2000) detected stx1 and stx2 in beef and milk samples with prevalence rates of 1.4 and 41.2%, respectively. Similar to our results, another study also found a higher prevalence rate of stx2 (20%) than stx1 (3%) in human clinical cases (Ostroff et al., 1989). This is also supported by the findings of Oporto et al. (2008) who found that E. coli O157 isolates carried stx2 and eaeA but only a few isolates had stx1. However, Himi et al. (2015) did not find stx1 in all examined E. coli serogroups. It is well known that stx2 is 1000 times more cytotoxic than stx1 and therefore it is associated with many diseases in humans (Mir et al., 2016).

Some animals harbor the Shiga toxin-producing E. coli O157 serotype in their intestine as a part of normal microflora (Fairbrother and Nadeau, 2006, Hammerun and Heuer, 2009, Ibekwe et al., 2011) and therefore these animals could act as a reservoir for E. coli O157 with a high possibility for human infection (Ferens and Hovde, 2011, Yan et al., 2011). This serotype can transmit from an animal into humans through consumption of contaminated and raw food and milk (Karch et al., 2005, Doma et al., 2020) causing severe diseases including hemorrhagic diarrhea and renal failure which can lead to deaths especially in young children, old and immunocompromised...
patients (Bandyopadhyay et al., 2011, Shahreza et al., 2017). Although *E. coli* O157 is not pathogenic to animals, an outbreak of fatal meningoencephalitis and septicemia in one-month-old goats caused by this serotype has been recorded (Filioussis et al., 2013).

**CONCLUSIONS**

The presence of Shiga toxin-producing *E. coli* O157 in fecal and raw milk samples of cattle threatens public health as cattle could serve as the main reservoir for transmission of this pathogenic serotype to humans. This is of particular importance because *E. coli* O157 is a member of cattle microflora that rarely cause diseases to these animals and so the possibility of transmission to humans (via fecal-oral route) is higher. Eating raw food or milk contaminated with cattle feces containing *E. coli* O157 could lead to STEC foodborne diseases in humans. Thus, attention should be given to biosecurity control measures in cattle farms to prevent the transmission of *E. coli* O157 from animals to humans. Additionally, *E. coli* O157 had multiple extended-spectrum β-lactamase genes that showed a remarkable resistance to some antibiotics commonly utilized in animal farms. Hence, many investigations are required to choose appropriate antibiotics and avoid misuse of antibiotics and subsequently reduce the spread of antibiotic resistance genes between different bacterial populations.

**CONFLICT OF INTEREST**

The authors declare that there is no conflict of interest.
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