The authors investigated the possibility of the presence of VTEC strains in improperly pasteurized milk samples. A total of 64 *Escherichia coli* strains were isolated from 135 pasteurized milk samples originating from the same producer. The examined isolates contained 29 haemolysin-, 9 colicin- and 5 aerobactin-producing strains, but the investigations concerning heat-resistant and heat-sensitive toxins gave negative results.

Six O128-type *E. coli* strains exerted a cytotoxic effect on the VERO cell line; 5 of them contained H12 antigen, while one could not be typed. Four of the 6 verocytotoxin-producing strains belonged in phage group 20, one in phage group (2)(3)(7), and one in phage group 4; four strains were of B3, one of A1, and one of A1(A2) phage type.

Because of a technical failure the milk was pasteurized at 69 ºC for 15 s, which is 2 ºC less than required. The results underline the importance of the appropriate pasteurization temperature, as otherwise the milk may contain verocytotoxin-producing *E. coli*, which is a potentially great hazard for public health.

**Keywords:** *Escherichia coli*, verocytotoxin, isolation, pasteurized milk samples, low pasteurization temperature

Verocytotoxin-producing *Escherichia coli* (VTEC), which is also known as shiga toxin-producing or enterohaemorrhagic *E. coli*, is an important emerging pathogen worldwide (Caprioli et al., 2006; Sakuma et al., 2006). The VTEC strains cause diarrhoea, haemorrhagic colitis (HC), and haemolytic uremic syndrome (HUS) (Lynn et al., 2005; Nguyen et al., 2006; Vali et al., 2007).

The VTEC isolates that cause human infections belong to the large number of O:H serotypes. *E. coli* O157:H7, the most prevalent serotype, is associated with large outbreaks and also sporadic cases of HC and HUS in many countries. *E. coli* O157:H7 was first recognized as a pathogen in 1982, during the investigation of an outbreak of HC (Riley et al., 1983). Infections with other non-O157 VTEC serotypes have been increasingly reported in many countries. More than 100 different non-O157 VTEC serotypes have been isolated from human infections. Unlike O157:H7, outbreaks caused by non-O157 VTEC have rarely been
reported, and these strains have been more frequently associated with sporadic cases of diarrhoea, HC and HUS (Herpay et al., 1994; Vaz et al., 2004).

The European Centre for Disease Prevention and Control (ECDC) has a Europe-based international surveillance network for the enteric infections. The VTEC database has been in existence since 2000. According to the recent data the total number of VTEC cases reported in the third quarter of 2007 was 594 from 16 countries. The most commonly identified serogroup was E. coli O157, which represented the majority of all reported serogroups (65%). Phage types 8, 32 and 4 were reported most frequently in the third quarter of 2007. HC was isolated more frequently in cases with VTEC O157 infections, compared to non-O157 infections, while HUS was as common in O157 cases as among non-O157 cases (Denny et al., 2008).

Verocytotoxins 1 and 2 (Vtx1 and Vtx2) are cardinal virulence factors of VTEC strains (Nguyen et al., 2006). Cattle are the principal reservoir for these organisms (Aidar-Ugrinovich et al., 2007; Mora et al., 2007; Varela-Hernández et al., 2007). Timm and co-workers (2007) investigated faecal samples from beef cattle to determine the prevalence of VTEC: 39% of the animals harboured at least one VTEC strain.

The literature data indicate four principal routes of human infection for VTEC: (a) direct contact with infected animals; (b) person to person transmission; (c) foodborne transmission; and (d) transmission through the environment (e.g. contact with environments such as pastures to which infected animals have had access). The infectious dose can be as low as 10 bacteria (European Commission, 2003). The major routes of infections are the consumption of raw milk and the contamination of well-water with agricultural runoff.

In a conference in Rome regarding the pathogenesis of VTEC infection, it was concluded that factors involved in the survival and transmission from animal reservoirs should be better determined (Caprioli et al., 2006). Accordingly, we have investigated the possibility of the presence of VTEC strains in pasteurized milk samples. We examined the serotypes, virulence factors and sensitivities against antibiotics and antibacterials of the isolated VTEC strains.

1. Materials and methods

1.1. Milk samples

On three occasions, 45 one-litre packages of milk samples with 3.6% fat content originating from the same producer were examined for the presence of E. coli.

1.2. Composition and preparation of media

Brilliant green bile lactose broth (BBL) medium and a synthetic medium designated X broth (Szita et al., 2003) as well as crystal violet neutral red bile lactose agar (VRBL) were applied for the detection of bacteria. The composition and preparation of BBL broth were described in ISO/DIS Standard (1979) and those of X broth according to Hungarian Patent (1987). The composition of X broth: ammonium sulphate (1 g l⁻¹), lactose (4 g l⁻¹), potassium hydrogen phosphate (3 g l⁻¹), magnesium sulphate × 7H₂O (0.005 g l⁻¹). Sterilisation at 121 °C for 20 min was carried out on 10 ml aliquots in tubes with Durham tubes. The composition and preparation of VRBL agar were described in ISO/DIS (1979).
1.3. Investigation of milk samples

One ml milk samples were inoculated into nine ml X broth enrichment medium. The enrichment medium was incubated at 30 °C for 48 h before 0.1 ml was plated onto VRBL agar. VRBL plates were incubated at 37 °C for 24 h. Five colonies were selected from the VRBL agar plates for identification.

1.4. Identification and investigation of isolated bacteria

For the identification of bacterial strains, the IMVEC biochemical tests were performed (indole production, methyl red reaction, Voges-Proskauer test, Elkman test and citrate utilization test). Computer-based biochemical identification with the Rapid API 20 E and Rapid ID 32 E (BioMerieux) tests (ATB Expression instrument) and a detailed computer-based analysis identified the E. coli strains.

Isolates identified as E. coli were screened for the presence of Shiga toxins.

1.5. Preparation of Vero cells and cytotoxic assay

Vero cell monolayers were prepared in 24 well microtitre trays and the cytotoxicity assay carried out according to Brooks and co-workers (1997).

Erlenmeyer flasks, 250 ml, containing 20 ml of Trypticase soy broth were inoculated by investigated E. coli strains and mechanically shaken at 37 °C. After 20 to 24 h, the cultures were centrifuged at 17,000 g for 30 min. The supernatants were filtered using 0.45 μm membrane filters (Millipore Corp., Bedford, Mass.) and stored at 4 °C until assayed.

The Vero cell culture assays were performed with nearly confluent cell monolayers grown in plates with 24 wells. At the time of assay, the growth medium was changed (0.5 ml per well), and 50 μl of undiluted culture filtrate was added. For controls, cultures received PBS or Tripticase soy broth. The cells were incubated at 37 °C in 5% CO₂ atmosphere, and after 24 and 48 h incubation the morphological changes in the cells were observed with a phase-contrast inverted microscope (Blanco et al., 2003). Cytotoxic activity was neutralised by using polyclonal antibodies against SLT-I and SLT-II, according to Konowalchuk and co-workers (1977).

The strains were serotyped via O, H and K antigens (Kauffmann-Knipschildt-Vahline scheme), and phagotyped. The production of haemolysin, colicin, aerobactin, heat-resistant (ST) and heat-sensitive (LT) toxins, furthermore the antibiotic sensitivity of the isolated strains were examined as described elsewhere (Herpay et al., 1994).

2. Results and discussion

Altogether 64 E. coli strains were isolated from the 135 milk samples in the two media. The strains isolated from each E. coli-infected sample belonged to serotypes O9, O19 and O128; furthermore 2 samples also contained a strain of the O3, and one-one sample strains of the O21, O37, O40 and O159 serotypes. Twenty-nine strains were haemolysin-, 9 colicin- and 5 aerobactin-producing, but the isolated bacteria did not produce ST or LT toxins.

Six of the O128 E. coli strains isolated from the milk samples exerted a cytotoxic effect on the Vero cell line. Five of them contained H12 antigen, but one could not be typed. Four strains belonged to phage group 20, one to phage group 4, and one to phage group (2)3(7). Four strains were of B3, one of A1, and one of A1(A2) phage type (Table 1).
Table 1. Serotypes and phage types of verocytotoxin-producing *Escherichia coli* strains

<table>
<thead>
<tr>
<th>Milk samples</th>
<th>O antigen</th>
<th>H antigen</th>
<th>K antigen</th>
<th>Phage group</th>
<th>Phage type</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>128</td>
<td>12</td>
<td>–</td>
<td>(2)3(7)</td>
<td>A1(A2)</td>
</tr>
<tr>
<td>2</td>
<td>128</td>
<td>12</td>
<td>–</td>
<td>20</td>
<td>B3</td>
</tr>
<tr>
<td>3</td>
<td>128</td>
<td>Nt</td>
<td>–</td>
<td>4</td>
<td>A1</td>
</tr>
<tr>
<td>4</td>
<td>128</td>
<td>12</td>
<td>–</td>
<td>20</td>
<td>B3</td>
</tr>
<tr>
<td>5</td>
<td>128</td>
<td>12</td>
<td>–</td>
<td>20</td>
<td>B3</td>
</tr>
<tr>
<td>6</td>
<td>128</td>
<td>12</td>
<td>–</td>
<td>20</td>
<td>B3</td>
</tr>
</tbody>
</table>

Nt: Non-typifiable

The 6 VTEC strains did not produce haemolysin, colicin, aerobactin, ST, LT (Table 2) and were sensitive against Streptomycin, Chloramphenicol, Tetracycline, Neomycin, Kanamycin, Ampicillin, Gentamycin, Co-trimoxazole, Nitrofurantoin and Nalidixic acid.

Table 2. Characteristics of the six verocytotoxin-producing *Escherichia coli* strains

<table>
<thead>
<tr>
<th>Milk samples</th>
<th>Haemolysin</th>
<th>Colicin</th>
<th>Lysogen</th>
<th>Aerobactin</th>
<th>Heat-resistant toxins (ST)</th>
<th>Heat-sensitive toxins (LT)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>2</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>3</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>4</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
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<tr>
<td>5</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>6</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
</tbody>
</table>

Investigation of the method of pasteurization applied by the milk company revealed that, due to a technical failure, the applied temperature was 69 ºC, which is 2 ºC less than prescribed.

Since VTEC can be present in the intestinal content and faeces of dairy cows (Timm et al., 2007), the faecal contamination of raw milk during its collection on dairy farms is recognized as a major VTEC transmission route. Due to the contamination of raw milk and/or improper processing, cow’s milk has been implicated in foodborne outbreaks. Wachsmuth and co-workers (1997) investigated outbreaks of VTEC O157:H7 in the USA from 1982 to 1995 and reported that raw milk was responsible for 5% of the outbreaks.

Quinto and Cepeda (1997) investigated the incidence of toxigenic *E. coli* in soft cheese made with raw milk or pasteurized milk. One VTEC strain of the O2 serotype was isolated from 221 samples of cheese produced from raw milk. No VTEC strains were detected in 75 cheese samples made from pasteurized milk. Our study demonstrated that the temperature during the pasteurization process was 2 ºC less than required, which was probably the reason why we were able to isolate 6 VTEC strains from 3 of the examined 135 milk samples.

VTEC O157 is most commonly associated with human diarrhoeal disease. The non-O157 VTEC serogroups are most often O26, O103, O111 and O145 (European Commission, 2003; Caprioli et al., 2006). The types of VTEC strains most frequently isolated from the faeces of
cattle are O79:H14, O113:H21 and O178:H19 (TIMM et al., 2007). We were not able to detect the strains listed above from the cow’s milk samples. According to Váz and co-workers (2004), the number of non-O157:H7 type VTEC strains isolated from human infections is currently increasing, and is now higher than 100.

3. Conclusions

In the course of our study, we isolated 5 O128:H12 and one O128:H non-typhi-able VTEC strains from the milk samples. We conclude that following a pasteurization process at 69 ºC for 15 s milk may contain VTEC, considered to be a potential source of infections for human beings.

References


