INFORMALLY RAW MILK AND KAREISH CHEESE INVESTIGATION ON THE OCCURRENCE OF TOXIGENIC ESCHERICHIA COLI IN QENA CITY, EGYPT WITH EMPHASIS ON MOLECULAR CHARACTERIZATION

Sabry A. Hassan* and Laila M. Elmalt**
*Department of Microbiology and **Department of Food Hygiene
Faculty of Veterinary Medicine, South Valley University, Qena, Egypt

ABSTRACT:

The informally raw milk and Kareish cheese sold in Qena city (Upper Egypt) were analyzed to determine the presence of toxigenic E. coli. The isolates were screened for the presence of verotoxigenic E. coli (VTEC) and enterotoxigenic E. coli (ETEC) by Polymerase Chain Reaction (PCR). Bio-resistance to antimicrobial agents was evaluated by the disk diffusion method. E. coli were recovered from 38 (76%) of raw milk and 11 (47.8%) of Kareish cheese samples. Three (6.1%) of the E. coli isolates were VTEC and none of them had eaeA gene encoded a pathogenicity island typical of E. coli O157:H7 (EHEC). PCR of enterotoxins showed that only one isolate carried LT enterotoxins of ETEC. Bio-resistance was frequently observed to nalidixic acid (42.9%), ampicillin (32.7%), tetracycline (22.4%), trimethoprim–sulfamethoxazol (14.3%), ciprofloxacin (4.1%) and cefoxitin (2.0%). Results suggested a possibility of potential public health threat of E. coli originating from raw milk sources.

INTRODUCTION:

Markets and consumers for raw milk and their products have existed in many parts of the world. Being a highly nutritious medium, therefore many bacteria including spoilage and pathogenic bacteria can grow and propagate in it. Generally, bacteria in the milk can occur through colonization of the teat canal or an infected udder (clinical and subclinical mastitis) or gets contaminated at various stages be it from the animal, milker (manual as well as automated), extraneous dirt or unclean process water (Gruetzmacher and Bradley, 1999; Hayes et al., 2001).

Several studies have identified milkborne pathogens including Shiga-toxin producing Escherichia coli (STEC) in farm bulk tank milk (BTM) (Moustafa et al., 1983; Lovett et al., 1987; McManus and Lanier, 1987; Rohrbach et al., 1992; O’Donnell, 1995; Rahn et al., 1997; Steele et al., 1997).

Many microorganisms can get access to milk and its products, among these is E. coli. which is often used as a marker organisms. Recovery and counting of Escherichia coli is
used as reliable indicator of fecal contamination and indicate a possible presence of enteropathogenic and/or toxigenic microorganisms which constitute a public health hazard. Escherichia coli is one of the main inhabitants of the intestinal tract of most mammalian species, including humans and birds. Most E.coli are harmless, but some known as pathogenic bacteria causing severe intestinal and extraintestinal diseases in man (Kaper et al., 2004). These potentially harmful E.coli are classified into categories based on the production of virulence factors and on the clinical manifestations that they cause. In addition to the presence of E.coli denoting fecal pollution, the presence of virulence-related genes in E.coli strains refer to the pathogenicity of the isolates. Previous studies documented the equation of some E.coli isolates from raw milk and its products for virulence markers (Klie et al., 1997; Jajarao and Henning, 2001; Holko et al., 2006; Paneto et al., 2007).

The present study aimed to investigate the occurrence of toxigenic E.coli isolates in raw milk and Kareish cheese using PCR assay and monitor the isolates to bio-resistance to different antimicrobial agents.

**MATERIALS AND METHODS:**

Sample collection:

A total of fifty raw milk and twenty-three Kareish cheese samples were randomly collected from different shops and distributors in Qena city. Samples were delivered to the laboratory in a cool box and tested within 24 hr.

Isolation and identification of E.coli:

Raw milk and Kareish cheese samples were taken for bacteriological analyses to detect the presence of E.coli. Concerning cheese samples, 25g were dispensed into a sterile flask containing 225 ml of buffered peptone water and homogenized with lab stomacher. E.coli confirmation was achieved by colony morphology on eosin methylene blue agar (EMB-Scharlau, Spain, EU) and performing API 20E (bioMérieux-France). Ninety-four E.coli stains were recovered from 50 raw milk and 23 cheese samples, and one isolates from each samples was used for further studies.

Bio-resistance of the isolated E.coli to some antimicrobial agents:

The susceptibility of isolates to different antimicrobial agents was done by disk diffusion method using commercial disks (Bauer et al., 1966). The antimicrobial agents tested were the following: nalidixic acid (30 µg), ampicillin (10U), tetracycline (30µg), trimethoprim-sulphamethoxazol (25 µg), ciprofloxacin (5 µg), cefoxtin (30 µg), amikacin (30 µg), imipenem (10 µg) and netilmicin (30 µg).

Polymerase chain reaction (PCR):

Bacterial strains were overnight grown in trypticase soy agar (TSA-Scharlau, Spain, EU) at 37°C. One colony was suspended in 100 µl of sterile distilled water. After boiling the suspension for 10 min, the supernatant was used as a template for PCR. Gene regions coding for the following pathogenic properties were amplified for each bacterial isolate: heat-labile toxin (LT), heat-stable toxin (ST), Shiga-like Toxin 1 and 2 (stx1, stx2), and enteropathogenic attachment and effacement (eaeA) using specific primers. Specific primers and amplification conditions for the different pathogenic gene coding regions were employed as previously described (Brian et al., 1992; China et al., 1996 and Matar et al., 2002). Details are shown in Table (1). For cycling, a PXE-0.5 thermal cycler (THERMO, Electron Corporation, Milford, MA, USA) was used. Amplified gene products were verified by gel electrophoresis (2% agarose) at 120 V for 40 min and visualised under ultraviolet light.
Table (1): Sequences and predicted size of PCR amplification products of the oligonucleotide primers used

<table>
<thead>
<tr>
<th>Pathogenic factor</th>
<th>Primer Sequences</th>
<th>Predicted Size (bp)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Shiga like toxin 1 (stx1)</td>
<td>aaatgcctgctacacttttgcccttgctcagcattcgttgactacttctggcattctggcaactcgcgatgca</td>
<td>366</td>
<td>Brian et al., 1992</td>
</tr>
<tr>
<td>Shiga like toxin 2 (stx2)</td>
<td>cgatcgtcactcactggtttcatca</td>
<td>282</td>
<td>Brian et al., 1992</td>
</tr>
<tr>
<td>Enteropathogenic attachment and effacement (eaeA)</td>
<td>agagcttcgtcacatgtgagcttcgtcacatctgctcaccagagga</td>
<td>579</td>
<td>China et al., 1996</td>
</tr>
<tr>
<td>Heat labile toxin (LT)</td>
<td>tcctgatgtaaagtagggcatactgattgccgcaat</td>
<td>320</td>
<td>Matar et al., 2002</td>
</tr>
</tbody>
</table>

RESULTS AND DISCUSSION:

*E. coli* is not only regarded as an indicator of faecal contamination but more likely as an indicator of poor hygiene and sanitary practices during milking and further handling. *E. coli* was isolated in 38 (76.0%) out of the 50 tested raw milk samples and 11 (47.8%) out of 23 Kareish cheese samples (Table 2). Milk can be easily contaminated by infected food handlers who practice poor personal hygiene or by water containing human discharges. Higher prevalence of *E. coli* was reported by many authors. In Egypt, Aly and Galal, (2002) showed the presence of *E. coli* in raw milk and the number reduced in the heat treated one. In India, the raw milk and products were heavily contaminated by *E. coli* (Soomro et al., 2002). In South Africa, Lues et al. (2003) detected a higher percentage of *E. coli* in raw milk. In Malaysia, Chye et al. (2004) indicated that 90% of the examined raw milk was contaminated by coliform bacteria and 65% were *E. coli* positive.

PCR showed that three isolates (6.1%) carried stx2 gene, and one isolate (2.0 %) of stx1 gene (Table 2), a value much higher than registered in Spain (0.4%) by Quinto and Cepeda, (1997), in Ontario (0.87%) by Steel et al. (1997), and in Germany (3.9%) by Klie et al. (1997). Meanwhile, was similar Paneto et al., (2007) who reported 6% in raw milk cheese in Brazil. In the other hand, less than 13% reported by Vernozy-Rozand et al. (2005) in French cheese. The results showed that, three of the four isolates of *E. coli* encoded for Shiga-Toxin 2 gene, while one strain encoded for Shiga-Toxin 1 gene and none of Shiga-Toxin carried strains hat eaeA gene encoded a pathogenicity island typical of *E. coli* O157:H7 (EHEC). On the contrary, Montenegro et al. (1990) reported that most of the STEC isolates of bovine origin encoded for Shiga-Toxin 1 gene. STEC have been associated with human disease. Foods of animal origin including raw milk have been implicated as important vehicles for STEC infections in humans. PCR of heat labile enterotoxins encoded for ETEC showed that only one of the tested strains carried LT gene (Table 2). Frank et al. (1984) reported the presence of 3.2% of ETEC strains in milk and milk products. Paneto et al. (2007) showed that only one isolate carried the LT-II gene while the ST gene was not found. ETEC are responsible for diarrhea in children.

Most frequent resistance was observed to the following antimicrobials: nalidixic acid (42.9%), ampicillin (32.7%), tetracycline (22.4%), trimethoprim-sulfamethoxazol(14.3%), ciprofloxacin (4.1%) and cefoxitin (2.0%) (Table 3). Paneto et al. (2007) examined VTEC strains from raw milk cheese, and similarly reported a high antimicrobial resistance to different antimicrobial agents and some of them were similar to those found in this study.
Resistance to at least one or more of tested antimicrobial agents was found in 42.9% of the examined isolates. A much higher resistance was observed in 83% of *E. coli* isolated from raw milk cheese in Brazil (Paneto et al. 2007). The high level of resistance may be a consequence of the abusive uses of antimicrobials in animal therapeutics as well as in food additives used to promote animal growth.

Table (2): Frequencies of isolation of *E. coli* and occurrence of pathogenic coding genes of *E. coli* isolated from informal raw milk and Kareish cheese marketed in Qena city, Egypt

<table>
<thead>
<tr>
<th>Sample source (n = number of samples)</th>
<th>Number of (%) <em>E. coli</em> isolates</th>
<th>Frequency of occurrence of pathogenic coding genes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>stx1</td>
</tr>
<tr>
<td>Raw milk (n=50)</td>
<td>38 (76.0%)</td>
<td>1 (2.6 %)</td>
</tr>
<tr>
<td>Kareish cheese (n= 23)</td>
<td>11 (47.8%)</td>
<td>-</td>
</tr>
<tr>
<td>Total (n = 73)</td>
<td>49 (67.1%)</td>
<td>1 (2.0%)</td>
</tr>
</tbody>
</table>

Table (3): Antimicrobial susceptibility testing of 49 *E. coli* isolates from raw milk and Kareish cheese

<table>
<thead>
<tr>
<th>Antimicrobials</th>
<th>No. of resistance</th>
<th>% of resistance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nalidixic acid (NALX)</td>
<td>21</td>
<td>42.9</td>
</tr>
<tr>
<td>Ampicilin (AMPC)</td>
<td>16</td>
<td>32.7</td>
</tr>
<tr>
<td>Tetracyclin (TET)</td>
<td>11</td>
<td>22.4</td>
</tr>
<tr>
<td>Trimethoprim-sulphamethoxazol (SMX/TMP)</td>
<td>7</td>
<td>14.3</td>
</tr>
<tr>
<td>Ciprofloxacin (CTPX)</td>
<td>2</td>
<td>4.1</td>
</tr>
<tr>
<td>Cefoxtin (FOX)</td>
<td>1</td>
<td>2.0</td>
</tr>
<tr>
<td>Amikacin (AMK)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Imipenem (IMIP)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Netilmicin (NET)</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

Figure (1): PCR amplicons of pathogenic coding genes determined in *E. coli* isolates from raw milk and Kareish cheese (a) stx1 366bp; (b) stx2 282bp; (c) LT 320bp. Lane M: 1Kb ladder, lane 1: test sample
CONCLUSION:

Results clearly indicated that microbial quality and safety of raw milk and Kareish cheese produced by local farmers and distributors was unsafe. The presence of faecal indicator organism not only indicates the poor hygiene but also itself may be pathogenic. The pathogenic bacteria such as *E. coli* may pass to the milk; this suggests that raw milk should be considered a vehicle for the transmission of potentially pathogenic bacteria.

Acknowledgments:

Prof. Y.A. Gherbawy (molecular biology unit, Botany department, Faculty of Science, South Valley University, Qena, Egypt) for use the lab facilities and to Prof. Awad-Masalmeh (University of Veterinary Medicine, Vienna, Austria) for *E. coli* (VT1-VT2- eaeA) and *E. coli* (LT).

REFERENCES:


تعيين حدوث بكتيريا القولون المعدي المفرزة للسموم في الألبان الطازجة والجبنة الفريش في مدينة قنا- مصر مع التشخيص الجزيئي

صبري عبد الرجال حسن*، ليلي مصطفى كامل الملط**

**KESLEAYIF BB425**

KESLEAYIF BB425

-42-