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

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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 hat 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 cefoxtin (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 methelene 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 oligonuleotide primers used

Pathogenic factor	Primer Sequences	Predicted Size (bp)	Reference
Shiga like toxin 1 (stx1)	aaatcgccattcgttgactacttct tgccattctggcaactcgcgatgca	366	Brian *et al.*, 1992
Shiga like toxin 2 (stx2)	cgatcgtcactcactggtttcatca ggatattctccccactctgacacc	282	Brian *et al.*, 1992
Enteropathogenic attachment and effacement (eaeA)	aggcttcgtcacatgtg ccatcgtcaccagagga	579	China *et al.*, 1996
Heat labile toxin (LT)	tctcattgtgcatacggagc Ccatactgattgccgcaat	320	Matar *et al.*, 2002

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 (Table2). 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 stx*2* gene, and one isolate (2.0 %) of stx1 gene (Table2), 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 (Table2). 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 cefoxtin (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.

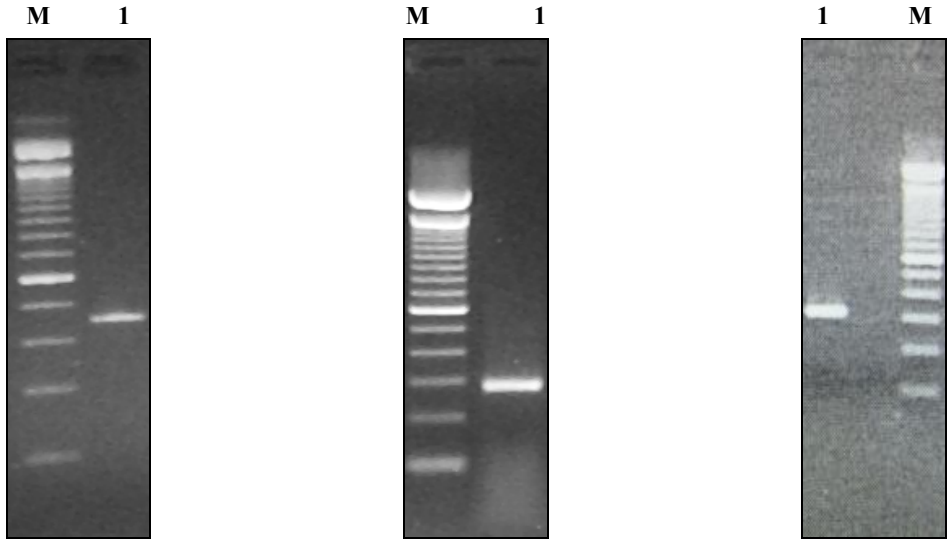
Resistance to at least one or more of tested antimicrobial agents was found in 42.9% of the examined isolates. A much higher resistance was observed in 83% of *E.coli* isolated from raw milk cheese in Brazil (Paneto *et al.* 2007). The high level of resistance may be a consequence of the abusive uses 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

Sample source (n = number of samples)	Number of (%) *E.coli* isolates	Frequency of occurrence of pathogenic coding genes			
		stx1	stx2	eaeA	LT
Raw milk (n=50)	38 (76.0%)	1 (2.6 %)	2 (5.3%)	-	1 (2.6%)
Kareish cheese (n= 23)	11 (47.8%)	-	1 (9.1%)	-	-
Total (n = 73)	49(67.1%)	1 (2.0%)	3 (6.1%)	-	1 (2.0%)

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

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



(a) stx1 **(b) stx2** **(c) LT**

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.

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

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***قسم الميكروبيولوجى، ** قسم المراقبة الصحية على الأغذية**

كلية الطب البيطرى – جامعة جنوب الوادى – مصر

تم إجراء دراسة بكتيريولوجية على بعض الألبان والجبن القريش التى تباع في أسواق مدينة قنا – مصر وذلك لتعيين مدى وجود بكتيريا القولون المعدي المفرزة للسموم. تم إجراء تعريف للعزلات المختارة إلى مستوي النوع، وكذلك تعيين الجينات المسئولة عن الضراوة والأمراضية باستخدام تفاعل البلمرة. تم تقييم مقاومة تلك العزلات لمختلف المضادات الميكروبية. أشارت النتائج بشكل عام إلى عزل نسبة عالية من ميكروب القولون المعدي، مما يدل على انخفاض الجودة. أظهرت نتائج تفاعل البلمرة الكشف عن وجود بعض الجينات المسئولة عن إحداث تلك العزلات لأمراض، وكذلك مقاومة بعض العزلات لفعل المضادات الميكروبية المختلفة. أوصت الدراسة باحتمالية خطورة استهلاك تلك الألبان الطازجة ومنتجاتها (الجبن القريش) على الصحة العامة.