

Isolation and Identification of toxigenic strains of *E.coli* from local cheese and evaluate their resistance to antibiotics

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Abstract

Raw milk cheese is considered a risk for food borne shiga-toxin-producing *E.coli* (STEC) contamination. The aim of the study is to isolation and identification of isolated strains by using serological and molecular methods. In this study 60 raw milk cheese samples were collected from different local of Baghdad city (each 10 sample from each region of Baghdad) such as (Algadedda Baghdad, Gesser Diyala, Fudhaliyah, sader city, Kadhimiyyah area and Rashid district). The isolated samples were cultured in selective media for identification and biochemical tests by using API 20 E and also by PCR technique for the presence of (STEC) by using *bfp* gene markers (*stx1*, *stx2*). The results showed the presence of *E.coli* 0.26 haemorrhagic type (EHEC) and the Enteropathogenic strain of *E.coli* (0.86, 0.127, 0.128) and the Enteroaggregative strain of *E.coli* (0.55). On the level of serological the isolates gave positive result that indicate presence of shiga toxin (*stx1*, *stx2*) in raw milk cheese. The result also showed multiple resistant of certain antibiotic (Amicillin 100%, Nalidixic acid and tetracycline and Gentamicin 80%, while was observed sensitive to Amikacin 100% and trimethoprim and cefotaxin 80).

Keywords: *Escherichia coli* strains, toxin, cheese, antimicrobial resistance

عزل وتشخيص سلالات *E.coli* المنتجة للسموم من الجبن المحلي ودراسة مقاومتها للمضادات الحيوية

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الخلاصة

يعتبر جبن الحليب الخام خطراً لنقله سموم بكتريا الايكولاي . فكرة الدراسة عزل وتشخيص هذه السلالات باستخدام الفحوصات السيرولوجية والجزيئية . في هذه الدراسة تم اخذ 60 عينة من جبن الحليب الخام من 6 مناطق متفرقة من بغداد بواقع 10 عينات من كل منطقه (بغداد الجديدة , جسر ديالى , الفضيلية , مدينة الصدر , الكاظميه , علوة الرشيد) وتم العزل على اوساط انتخابية وتشخيصها كيميوكيويا باستخدام PCR وبتقنية Api20 E وظهرت النتائج وجود الجين *bfp* 0.26 والمنتج للسموم نوع 1, 2 , كما اظهرت النتائج وجود السلالة النمطية النزفية والمرضية 0.86 , 0.127 , 0.128 , والمتجمعه 0.55 . من جهة اخرى اظهرت النتائج مقاومة البكتريا للمضادات الحيوية , حيث اظهرت مقاومه للمضاد امبيسلين 100% وحامض النيلديكسك

والنتراسايبكلىن والجينتاماميسين 80% بينما اظهرت النتائج حساسية البكتريا اتجاه مضاد الاميكاييسين 100% والسيوفوتوكسين 80%.

Introduction

The production of local one of the most popular cheeses produced in Iraq. This soft white cheese is made from pasteurized or raw milk. It is characterized by a high water content (50.0%) and low pH (5.1-5.6) (Freitas *et al.*, 1993). Recovery and counting of *Escherichia coli* is used as an index of recent fecal contamination and indicate that other microorganisms of fecal origin may be present (Portaria..., 1997). After an outbreak of foodborne disease caused by *E. coli* strains (Marier *et al.*, 1973), the presence of these microorganisms in cheese acquired additional significance. Toxigenic *E. coli* of bovine origin has been classified into five categories: Enterotoxigenic *E. coli* (ETEC), Enteropathogenic, *E. coli* (EPEC). Enterohemorrhagic, *E. coli* (EHEC). Enteroinvasive *E. coli* (EIEC) and Enteroaggregative *E. coli* (EAEC) (Quinto and Cepeda, 1997). The first group constitutes of the most important vectors of *E. coli* diarrhea and is considered the major cause of diarrhea in children in developing countries. It is also the most frequently etiological agent responsible for diarrhea (Decludt, B., Bouvet, P *et al.*, 2000). ETEC causes diarrhea by adhering to the intestinal mucosa by their unique colonization factors, producing either heat-labile enterotoxins (LT-I and LT-II), heat-stable enterotoxins (STa and STb), or both (Nataro and Kaper, 1998). Enteropathogenic *E. coli*, like ETEC and EPEC also causes diarrhea, but the molecular mechanisms of colonization and aetiology are different. EPEC lack fimbriae, ST and LT toxins, but they use an adhesin known as intimin to bind host intestinal cells. This virotype has

an array of virulence factors that are similar to those found in *shigella*, and may possess a shiga toxin (Bertin, *et al.* 2001). Adherence to the intestinal mucosa causes a rearrangement of actin in the host cell, causing significant deformation. EPEC cells are moderately invasive (i.e. they enter host cells) and elicit an inflammatory response. Changes in intestinal cell ultrastructure due to "attachment and effacement" is likely the prime cause of diarrhea in those. The most infamous member of this virotype is strain O157:H7 which causes bloody diarrhea without fever (Schuller, S., Frankel 2004). EHEC can cause haemolytic uraemic syndrome and sudden kidney failure. It uses bacterial fimbriae for attachment (*E. coli* common pilus, ECP), is moderately invasive and possesses a phage-encoded shiga toxin that can elicit an intense inflammatory response. Effect with Verocytotoxin (VT)-producing *E. coli* (VTEC) are a well recognized cause of severe disease in human beings (Fernandez, *et al.* 2006). While numerous outbreaks have been related to *E. coli* serotype O157:H7, several other VTEC serotypes have been associated with human diseases (Paton and Paton, 1998). Outbreaks and sporadic cases of illnesses were also traced to consumption of VTEC-contaminated cheese (Deschenes *et al.*, 1996). Enteroinvasive (EIEC) infection causes a syndrome that is identical to shigellosis with profuse diarrhea and high fever. Enteroaggregative (EAEC) named because they have fimbriae which aggregate tissue culture cells, EAEC bind to the intestinal mucosa to cause watery diarrhoea without fever. EAEC are noninvasive, they produce a hemolysin and an ST enterotoxin

similar to that of ETEC. Cattle are an important reservoir of toxigenic *E. coli*, and have been implicated as a source of *E. coli* that infect and cause disease in human beings (Hornitzky et al, 2002). The aims of the present study were to determine the presence of *E. coli* strains in soft cheese made of raw milk in Baghdad, and also to verify the resistance of *E. coli* strains to antimicrobial agents.

Aim of study

- 1- The present study gave an idea about the possibility of using serological and molecular technique to identify certain isolates from raw milk cheese and multiple resistance to certain antibiotic that used in the study. In addition to highlight potential occurrence of STEC in the cheese region of Baghdad that give indication of contamination and poisoning in the selected local Baghdad.
- 2- Using techniques API and Vitek in the diagnosis of *E. coli* strains isolated

MATERIALS AND METHODS

Sixty samples of soft cheese made of raw milk were purchased from local producers. All samples were kept at 4 °C in plastic bag information about dates of production and of assigned shelf lives were not presented. Samples were collected over a period of six months between February and July for 2013, and were analyzed for the day of purchasing. Samples were transported at (4-6°C) in ice boxes containing ice packs and were tested immediately after collection. Twenty five sample were blended with 225ml of nutrient broth for two min at normal speed, using a Stomacher lab blender and incubated at 37°C for 24h. 1ml of the nutrient broth culture was mixed

with 9ml of MacConkey broth and further incubated at 37°C for 24h. One loop of each tube was streaked on MacConkey agar deep red colonies were produced, as the organism lactose-positive. Four colonies from each plate with typical *E. coli* morphology were selected and examined by biochemical tests, including indole-positive (red ring) and methyl red-positive (bright red), but VP-negative (no change-colourless) and citrate-negative (no change-green color) (Koneman *et al.*, 1997). Five *E. coli* strains were isolated from sixty cheese samples, and one isolated from each cheese sample was used for further characterization except kadhimiya. Five samples were studied for the determination of O antigens, All *E. coli* colonies were tested for slide agglutination with commercial polyvalent and monovalent antisera and conformed by API 20E and Identification by VITEK-Compact 2. The antimicrobial susceptibility patterns of *E. coli* strains isolated from cheese was done by the disk diffusion method using commercial disks according to the guidelines of the National Committee for Clinical Laboratory Standards (Performance..., 2002), testing the susceptibility against 7 antimicrobial drugs. Antimicrobial agents tested, loaded on the disks were the following: ampicillin (AMP-10) amikacin (AMI-30) nalidixic acid (NAL- 30) cefotaxime (CTX-30) gentamicin (GEN-10) tetracycline (TET-30) trimethoprim (TRI-5). Usiry Mueller Hinton Agar. Bacterial strains were overnight grown in nutrient broth at 37°C. 100µl sample of the culture was centrifuged and the pellet was resuspended in distilled water. After boiling the suspension for 10min, the supernatant was used as a template for

PCR. The base sequences annealing temperatures and predicted sizes of the amplified products for the specific oligonucleotide primers used in this study. The amplified product was visualized by ethidium bromide staining after gel electrophoresis of 10µl of the final reaction mixture in 1.5% agarose.

RESULTS AND DISCUSSION

E. coli was isolated from five (8.3 %) out of the sixty tested cheeses. Five strains were isolated and were identified by the slide agglutination serological test using available polyvalent and monovalent antisera as

Table 1. Serogroup and Virulence factors of *Escherichia coli* isolates from cheese made of Raw milk in Baghdad (n= 5)

| <i>E.coli</i> Serogroup | N of isolates % |
|-------------------------|-----------------|
| 0.26 | 1 (20%) |
| 0.55 | 1 (20%) |
| 0.86 | 1 (20%) |
| 0.127 | 1 (20%) |
| 0.128 | 1 (20%) |

during the manufacturing process of soft Hispanic-type cheese (Kasrazadeh *et al.*, 1995). These findings indicate that food of animal origin may be a significant source of pathogenic species of *E. coli*. Result showed both isolated were multiple resistance to Ampicillin 100%, Nalidixic acid, tetracycline and Gentamicin 80%. While was observed sensitive to Amikacin 100% and trimethoprim and cefotaxim 80%. Most frequent resistance was observed to the following antimicrobials: nalidixic acid (40mm), tetracycline (31mm) and ampicillin (29mm). Zhao *et al.* (2001) examined VTEC strains from humans, animals and food, and reported a high antimicrobial sensitive to Amikacin (48mm), trimethoprim (48mm) and cefotaxim (33mm), and some of them were similar to those found in this study. Resistance to at least one of a series of tested antimicrobial agents

pertaining to serogroups O.26, O55, O86 and O127 .O.128 all commonly involved in human diseases (Nataro and Kaper, 1998). Similar results were reported by Araujo *et al.* (2002) who found that 97.0% of soft cheese samples from Rio de Janeiro, Brazil, contained *E. coli* of the same serogroups, among others. Serogroups O111 and O119 have been recognized as important pathogens in Brazil (Gomes *et al.*, 1991) and also have been isolated as STEC strains from diarrheic and non-diarrheic calves in Brazil (Leomil *et al.*, 2003).

PCR showed that isolate carried the VT2 gene, and isolate the VT1 gene, a value much higher than 0.4% registered by Quinto and Cepeda (1997) in soft cheese in Spain, but less than the 13.0% reported by Vernozy-Rozand *et al.* (2005) in French cheese. PCR of heat labile (LT-I and LT-II) and heat stable (STa and STb) enterotoxins showed that only one isolate carried the LT-II gene while the ST gene was not found. Frank *et al.* (1984) reported the presence of 3.2% of ETEC strains in milk and milk products. Soft and semi-soft cheese have been previously associated with disease outbreaks involving pathogenic strains of *E. coli* (MacDonald *et al.*, 1985; Deschenes *et al.*, 1996) which demonstrated that contamination occurs at some point during cheese production and processing. Experimental studies have already shown that *E. coli* O157 can survive

resistance. The high level of resistance may be a consequence of the abusive use of antimicrobials in animal therapeutics as well as in food additives used to promote animal growth.

was found in 83.0% of the examined isolates. Khan *et al.* (2002) reported resistance to one or more antimicrobials 49.2% of the VTEC strains in India, moreover, some of those strains showed multidrug-

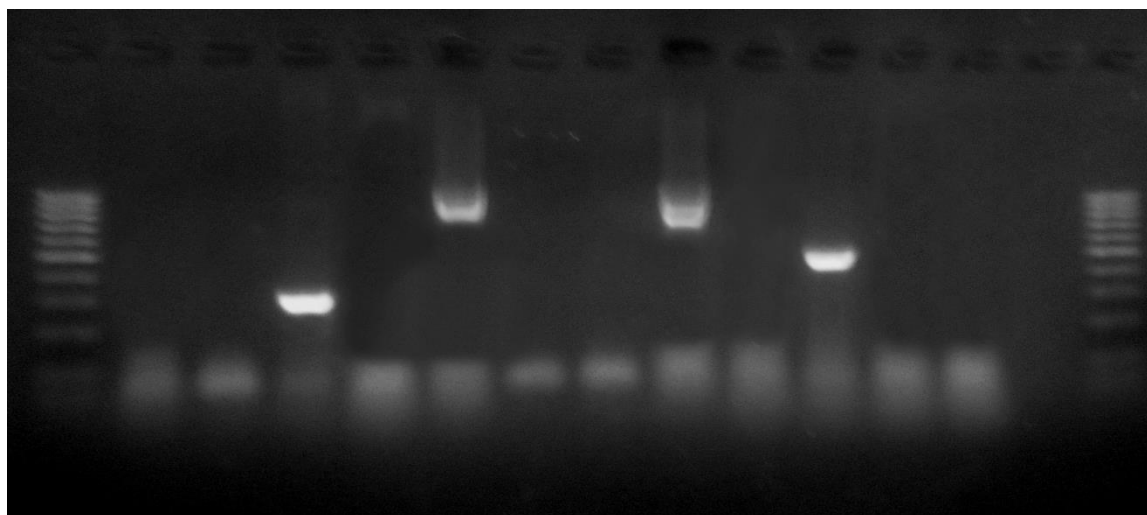


Figure 1. Gel electrophoresis of amplified *bfpA* (326bp), SLT I (894bp) and SLT II (478bp) from *E. coli* DNA isolated from cheese specimens. Agarose (1.5%), 5 V/cm for 2 hrs, stained with ethidium bromide and visualized on a UV transilluminator.

Lane 1 and 14 . 100 bp DNA ladder.

Lane 2-5. Amplified *bfp* gene of Serogroups 55, 86, 127 and control respectively.

Lane 6-10. Amplified SLT I gene of Serogroups 26, 55, 86, 128 and control respectively.

Lane 11-13. Amplified SLT II gene of Serogroups 55, 86 and control respectively.

Table 2. the table below shows sensitivity of the test *E.coli* strain measured in millimeter(mm) zone of inhibition According to (NCCL)

| 0128 | 0.127 | 0.86 | 0.55 | 0.26 | Inter medlate | Antibiotic |
|---------|---------|---------|---------|---------|---------------|----------------|
| R | R | R | R | R | 16-14 | Ampicillin |
| S 20 mm | S 25 mm | S 30 mm | R | S 37 mm | 15-11 | Trimethoprim |
| R | S 19 mm | R | R | R | 18-14 | Nalidixic Acid |
| S 29 mm | S 30 mm | S 30mm | S 33 mm | S 35 mm | 16-15 | Amikacin |
| R | S 22 mm | R | R | R | 19-16 | Tetracycline |
| S 26 mm | S 24 mm | S 30 mm | S 30 mm | R | 22-15 | Cefotaxine |
| R | R | S 22mm | R | R | 19-16 | Gentamicin |

Not. Resistant(R) .Sensitive(S) .zone of inhibition millimeter(mm) .Control (zero)
National Committee for Clinical Laboratory Standards (NCCL)

It is concluded that toxigenic *E. coli* may pass to the milk destined results of other authors (Quinto and Cepeda, 1997; Araujo et al., 2002; Vernozy-Rozand et al., 2005). They represent a health hazard and this suggests that soft cheese should be considered a vehicle for the transmission of potentially pathogenic bacteriato manufacture cheese, surviving in soft cheese made of raw milk, confirming the.

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