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Detection of Verotoxigenic *E. coli* O157:H7 in Raw Milk Using Duplex PCR in Basra City- Iraq

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Abstract

One hundred fifty milk samples were collected from three different markets in Basra city during a period extend of from November 2010 to March 2011. All samples were cultured in selective media to detect the presence of non-sorbitol fermenting colonies (NSF) 86 (57.34%) isolates were found non-sorbitol fermenting *E. coli*. Latex agglutination test was used to detect serotype O157:H7 in non- sorbitol fermenting isolates for 13 (27.08%) isolates. Multiplex PCR were done to all *E. coli* O157:H7 isolates and the result showed 7/150 (4.67%) from raw milk were positive to this test. All the multiplex PCR positive *E. coli* O157:H7 isolates were positive to VT1 gene which was observed in 100% except one isolates which was positive to (VT1and VT2) genes at 14.28% from 7 isolates of raw milk samples.

Keywords: Basra, *E. coli* O157:H7, milk, PCR

Introduction

Milk and its products are considered a kind of proteins, fats, salts rich nutrient with sweet taste, easy digested, cheep markets, and consumers for raw milk and their products have existed in many parts of the world. Milk is a highly nutritious medium for growth

and transmission of several types of microorganism especially *E. coli* O157:H7. Such contaminants may render the milk and its products unsafe to use and expose the consumers to risk of infection (USDA, 2009; Robert, 2008). In recent years, Since the identification of *E. coli* O157:H7 as a human pathogen in 1982 in Oregon and Michigan, others are now commercially available for the major VTEC serogroups (O157, O26, O111, O103 and O145) previously identified as being commonly associated with human disease and have become a very important milk-borne pathogen which constitute a public health hazard (Fratamico and Smith, 2006). *Escherichia coli* O157:H7 serotypes are identified as entero-haemorrhagic *E. coli* EHEC and categorized as verotoxin-producing *E. coli*. verotoxin is also known as shiga-like toxin, human and bovine *Escherichia coli* O157:H7 elaborates two potent phage encoded cytotoxins, known as Shiga-toxins *Stx1* and *Stx2* or verotoxins *VT1* and *VT2* (EFSA, 2007 and Jamshidi *et al.*, 2008). Detection methods for the isolation and identification of *E. coli* O157:H7 in food samples are categorized into two types conventional and rapid methods. These methods are based on cultural, serological, and biochemical properties of *E. coli* O157:H7. Selective agar media with inhibitors to reduce growth of unwanted species is done for the presumptive identification of organisms on the basis of distinct biochemical reactions and the isolates are further tested serologically for the presence of the O157 and H7 antigens using commercial available latex agglutination kits or antisera (Chow *et al.*, 2006). Multiplex PCR high sensitivity, specificity, its availability in many formats, rapid-screening tests have been used extensively for the identification and characterization of target bacteria in food samples, Including meat and dairy products based on immunological or nucleic acid technologies developed for food testing can provide results within hours (Ercolini *et al.*, 2004 ; Alarcon *et al.*, 2006).

Materials and Methods

 A total of one hundred fifty random raw milk was collected from local retail markets of three different markets of Basra city which were (market 1, market 2, and market 3). Fifty samples from each market were collected through period extended from November 2010 to March 2011.

Latex agglutination Test for *E. coli*O157:H7 was used for more specific identification of *E. coli* O157:H7 by using commercial kit (Wellcolex *E.coli* O157:H7, Remel) to detect the somatic antigen O157 and flagellar antigen H7.

Molecular Detection of verotoxine gene ((VT1 and VT2) gene by using Multiplex PCR technique was done by using commercially available DNA extraction and purification kit (Geneaid, USA). The purified DNA was detected by electrophoresis in 1% agarose gel with addition of ethedium bromide. Methylene blue stain added to the DNA sample and visualizes the DNA by U.V. light.
VT1a: GAAGAGTCCGTGGGATTACG   130 bp (Pollard *et al.,* 1990)

VT1b: AGCGATGCAGCTATTAATAA

VT2a: TTAACCACACCCACGGCAGT   346 bp (Pollard *et al.*, 1990)

VT2b: GCTCTGGATGCATCTCTGGT

The verotoxin genes were studied according to protocol of (Pollared *et al*., 1990). This was done by using customize primers. The PCR reaction mixture contains 5 µl of green master mix (contains bacterially derived *Taq* DNA polymerase, dNTPs, $MgCl_2$ and reaction buffer at optimal concentration for efficient amplification of DNA templates by PCR), 5 µl of purified bacterial DNA, 1 µl of each forward and reverse primers, then the volume completed to 20 µl by deionized water. All tubes were centrifuged in microcentrifuge for 10 seconds. The PCR tubes were transferred to the thermalcycler to start the amplification reaction according to specific program for each gene. The agarose gel was prepared according to the method of (Sambrook *et. al.*, 1989). Two concentrations of agarose gel were prepared (1% and 2%) as we needed. The concentration of 1% agarose was used in electrophoresis after DNA purification process, while 2% agarose was used after PCR detection. The results of the PCR were performed in post amplification process.10 μl from amplified sample was directly loaded in a 2% agarose gel containing 0.5 μl /25ml ethidium bromide with the addition of loading buffer and DNA size. Marker as standard in electrophoresis and the gel was run at 75 V. at 1 hr, then the products were visualized by UV transilluminator.   The results were analyzed statistically by chi-square test (SPSS, 11). (Niazi, 2000).

Results

According to the results of culturing on TC- SMAC, identification and biochemical conformation, 86 out of 150 tested samples analyzed 57.34% were NSFEC positive. The suspected colonies of *E. coli* on SMAC were small, circular and colorless with smoky center (1-2) mm in diameter. While on MacConkey agar, the colonies were pink in color (lactose fermenter), on Eosin–Methylene Blue (EMB) agar the colonies had metallic sheen appearance. Microscopic examination of Gram's stained to the suspected VTEC isolates revealed Gram negative small bacilli. Biochemical tests (IMViC, TSI, and cellobiose) of NSF colonies showed that 55.81%, 47.87% of raw milk, and soft cheese isolates were positive (*E. coli*) respectively (Table 1). All of the *E. coli* isolates were tested by latex agglutination test for the somatic O157 antigen. The results showed that 56.25% of *E. coli* isolates from raw milk were positive for O157 somatic antigen. The O157 positive isolates were tested for the flagellar H7 antigen. The Frequency of H7 antigen positive isolates were 27.08%.

Table (1) The Frequency of Biochemically Confirmed *E. coli* O157:H7 among *E. coli* isolates

No Samples	Nonsorbitol fermenter (NSF) (%)	No. of biochemically* positive (%)	O157 +ve (%)	H7 +ve (%)	O157:H7 +ve (%)
150	86 (57.34)	48 (55.81)	27 (56.25)	13 (27.08)	13 (27.08)

* =   IMViC pattern ++--, TSI & cellobiose tests

Distribution of *E. coli* O157:H7 Serotypes According to Period of the Study

 The results of the present study showed that the highest rate of *E. coli* O157:H7 isolation was in March (12.5%) followed by February (8.5%), December (8%), November (6.6%) and January (5%). There were no significant differences in the isolation rat according to the period of study, (Table. 2).

Distribution of *E. coli* O157:H7 Serotype from Raw Milk among the Different Regions of the Study.

 According to the results of isolation and identification of *E. coli* O157:H7. There were 13 samples out of 150 tested samples were positive for the above isolate. The percentage of isolates in raw milk were 53.84%. The high rate of isolation was observed in market 1 (57.14%) followed by market 2 and market 3, 50% for each one. There were no significant differences ($p > 0.05$) in the rate O157:H7 isolation among the regions of the study (Table3).

Table (2) The Distribution of O157:H7 in Raw Milk Samples According to the Period (month) of Collection.

Month	No. of samples	No. of positive O157:H7	%
November / 2010	30	2	6.6
December / 2010	25	2	8
January / 2011	20	1	5
February / 2011	35	3	8.5
March / 2011	40	5	12.5
Total	150	7	4.67
$X^2 = 1.248$ $(p > 0.05)$			

Table (3) Distribution of VTEC O157:H7 in *E coli* isolates according to the regions of the study

Market	No. of samples	No of *E. coli* O157:H7	%
Market 1	50	7	57.14
Market 2	50	2	50
Market 3	50	4	50
Total	150	13	53.84
$X^2 = 23.2$ $P > 0.05$			

**Distribution of PCR positive VTEC isolates in raw milk Samples**

 The DNA of all isolates was extracted and purified by using genomic DNA purification kit. Out of 27 *E. coli* O157 or O157:H7 isolates which were previously confirmed by biochemical and serological testing, 7 isolates (25.92%) were identified having VTEC

genes. The overall prevalence of PCR positive VTEC was 4.67% (7/150) of the tested raw milk. All the isolates which were positive for *E.coli* O157:H7 latex agglutination test were amplified to detect of *vt1* and *vt2* genes. *vt1* gene (about 130bp) was observed in percentage100 % (7/7) of the tested isolates while only one isolate of *E.coli* O157:H7 was observed having both genes (*VT1* and *VT2* (about 346bp) in a percentage 14.28 % (Table, 4 and figure 1).

Table (4): Distribution *VT1* and *VT2* genes in *E. coli* O157:H7 isolates from raw milk samples

PCR + ve VTEC No.	Genes					
	VT1	*%*	*VT2*	*%*	*VT1+VT2*	*%*
7	7	100	1	14.28	1	14.28

Figure (2): PCR amplification of *VT1*gene, 130bp (Lan 2-7) and *VT2* gene, 346bp (Lane 5). Lane 1&8 ladder
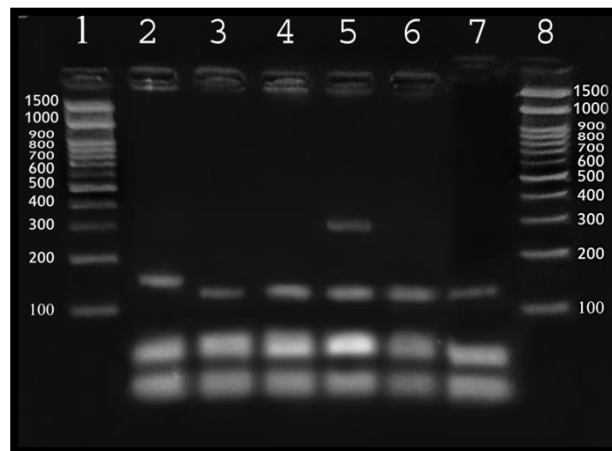
Discussion

The frequency of NSF isolates in raw milk samples (57.34%) was higher than the prevalence reported by Roopnarine *et al*., (2007) who recorded that NSF isolates from milk was 37.5%, also much higher than Daood, (2007) and Adesiyun *et al*., (2007) who showed that isolation rate was 10%, 14.2% respectively. The rate of milk positive isolates in the present study was 57.34% which agrees with the result obtained by Soomro *et al*., (2002) which was 57%. Other studies such as Abdul-Raouf and El-safey, (2003), Mansouri-Najand and Khalili, (2007) who mentioned much lower rates of NSF isolates in raw milk (5%, 4.46% respectively) in comparison with the rate in the present study.

The explanation of differences in isolation rate between this study and other studies may be related to the difference in the serotypes of NSF *E. coli* and to facilities techniques used in detection and diagnosis of this bacteria. In the present study NSF *E. coli* was isolated on selective enrichment (TSB-V) supplemented with vancomycin (De-Boer and Heuvelink, 2000). This medium permits the growth of *E. coli* and inhibit a wide range of contaminants including *Proteus spp.* Also it is necessary for the increment of bacteria to the level in which easily detected as low (10-100 cells) infection dose (Reissbrodt, 1998). On the other hand, the selective medium (TC-SMAC) in this study was supplemented with cefixime (0.05mg/L) and potassium tellurite (2.5mg/L). Those inhibited other enteric organisms which compete overgrow the targeted organism of the present study.

In the present study, the overall prevalence of *E. coli* O157:H7 latex agglutination test was 27.08%. This is concordant with the results showed by (Al-Aidi and Najim, (2009), Murinda *et al*., (2002) and Daood, (2007) which were 23.6%, 26.7%, 30.90% respectively, while it is much higher than results obtained by Al-Hasnawi, (2010), Karns *et al*., (2007), Belickova *et al* .,(2008) and Stephan *et al*., (2008) which were 11.1%, 0.23%, 1.02%, 5.0%, 2.5% respectively. In All these previous studies, the prevalence rates were less than the results in the present study, this indicated the presence of O157:H7 of tested samples included in the present study with higher prevalence due to unhygienic measurement that lead to higher contamination rates, suggested that spread of *E. coli* O157:H7 in the raw milk which were serve as a main source of infection and the risk of acquiring is high (Jamshidi *et al*., 2008). The prevalence of PCR positive for vt_1 of *E. coli* O157:H7 isolates from raw milk samples (4.67%) was concordant with the results obtained by Karns *et al*., (2007) and Fitzmaurice, (2003) which were 4.2% and 4.7%, respectively. On the other hand, the isolation rate in this study was much higher from that registered in Spain (0.4%) by Quinto and Cepeda, (1997), in Ontario (0.87%) by Steel *et al.,* (1997), in Egypt (1.10%) by El-Safey, (2001) and in Germany (3.9%) by Klie *et al.,* (1997). The high rate recorded in the present study can be attributed to the use of primers designed to target genes vt_1 and vt_2 genes which encoded for *Vt1*, *Vt2* toxins respectively and these two sets of oligonucleotide primer were used in multiplex PCR assay for the detection of *Vt* genes or as a means to increase sensitivity and specificity of this technique than other assays for the detection of VTEC O157:H7 in raw milk and soft cheese (Aslam *et al*., 2003). In the present study, the highest isolation rate of *E. coli* O157:H7 from raw milk cases obtained in March (7.5%) followed by February (5.71%). These results agree with the results obtained by Murinda *et al*., (2002), Spano *et al*., (2003), Rahimi *et al*., (2008) and Al-Aidi & Najim, (2009) who found that the highest isolation rate percentage. This is an increase in viability and survival of *E. coli* O157:H7 during the warm months of the year which was nearly from optimum temperature for growth than cold months. In contrast, a study in Scotland (Ogden *et al*., 2004) stated that the highest isolation rate was obtained in winter.

On other hand, according to the regions of study the highest isolation rate of O157:H7 from raw milk cases was recovered in market 1 (57.14%) followed by market 3 and market 2 (50%) for each one. The results showed that season, geographical location of the farmer villages, contaminated environmental conditions and unhygienic measures had an effect on the isolation percentage of *E. coli* O157:H7 (Spano *et al*., 2003).

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