Molecular detection of Stx1 and Stx2 genes in E. Coli O157:H7 isolated from Cow's and Buffalo's soft cheese samples in Babylon Province, using Multiplex PCR technique.

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Abstract
Colonies of E. Coli O157:H7 were isolated from 80 locally produced Cow's and Buffalo's soft cheese samples that were collected randomly at weekly intervals (5 samples/week) from various retail markets in different locations of Babylon province during two climatic periods (40 samples/species/season) where the first period was extended from the beginning of December 2015 to the end of February 2016 while the second period was extended from the beginning of July to the end of September 2016. The identification of E. Coli O157:H7 isolates were confirmed based on their cultural, biochemical and serological characteristics using the commercial latex agglutination test kit and by duplex PCR technique. Data revealed that there was a significant (P≤ 0.05) seasonal variation in the prevalence of E. Coli O157:H7 where all Cow's and Buffalo's soft cheese samples had significantly (P≤ 0.05) higher prevalence of E. Coli O157:H7 in summer season (50% and 40% respectively) than in winter season (25% and 15% respectively). It was found that all of the 80 bovine soft cheese samples had significantly (p<0.05) higher prevalence of E. Coli O157:H7 in summer (45%) than in winter (20%) seasons.

The results of the diagnosis of E.coli O157:H7 were confirmed by PCR assay which showed that, six culturing isolates serotype E.coli O157:H7 were positive by PCR assay and five of these isolates expressed gene of Stx1 whereas one isolate expressed gene of Stx2.

KEYWORDS: E. coli, PCR, Stx1 and Stx2 genes, Babylon.
Introduction
Since the identification of *E. coli O157:H7* as a human pathogen in 1982 (Fratamico and Smith,2006), *E. coli O157:H7* has become a pathogen of major concern for the food and dairy products because of its ability to cause severe illness,in particular,hemorrhagic colitis,hemolytic uremic syndrome and thrombotic thrombocytopenic purpura(Govaris et al.,2001;Maher et al.,2001). *E. coli O157:H7* is a member of a group of pathogenic *E. coli* strains, enterohemorrhagic *E. coli* (EHEC),Verotoxin producing *E. coli*(VTEC), Shigatoxigenic *E. coli* (STEC) which was isolated from individuals who developed bloody diarrhea and severe abdominal cramp(Yoon and Hovde,2008).Most of the food borne outbreaks of *E. coli O157:H7* have been associated with the consumption of foods originated from cattle, especially foods contaminated with cattle faeces,because *E. coli O157* has been found regularly in healthy cattle faeces,which is known to be an asymptomatic carrier (Öksüz et al.,2004).In 1999,over (11%) of the total number of reported cases of infections caused by *E. coli O157:H7* in England and Wales were due to dairy products (Vernozy-Rozand et al.,2005).Cheese made from unpasteurized milk is a potential vehicle for the transmission of *E. coli O157:H7* to the consumer.In Iraq,similar to other countries,domestic cheeses are still very popular which usually produced from raw milk with insufficient hygienic practices.A large amount of traditional cheeses are manufactured from raw milk and consumed freshly or after ripening in salty brine.Over the last several years, detection methods of STEC in foods have been significantly developed from culture-based methods into DNA-based and immune assays with each method having its strengths and weaknesses (Derzel et al.,2011).In addition,DNA based methods demand for specific laboratory equipment, may constitute a financial burden especially in developing countries.Rapid and reliable methods for the detection of *E. coli O157:H7* in different food products are needed to ensure food safety.The combination of rapidity,good sensitivity and specificity,and ease of performance has made PCR technology an appealing alternative to the cultural-based and immunological-based methods for pathogens detection in foods (Rendon et al.,2007).Diagnosis of the causative agent of diarrheas cannot depend only on the clinical features of the patients but requires proper diagnosis of the infectious agent in the laboratory (Allerberger et al.,2003).

Advantages of multiplex PCR assays,in which two or more DNA regions are co-amplified in one reaction,are lower cost and less time to obtain results.(Wang et al.,2006).In the previous study,PCR assays using two combinations of primers were designed for the detection of *E. coli O157:H7* in imported beef and lettuce,by detection of genes present in a limited number of serotypes. For example, the inclusion of the *E. coli O157:H7* O antigen marker rfbEO157 limited the detection of strains to O157 sero groups (Barletta et al.,2009).The aim of this study was identification of *E. coli O157 H7* from the locally produced homemade soft cheese.

كلمات المفتاحية:الاشريشيا القولونية,E. coli O157:H7 ,الجبن الطري, E. coli O157:H7 , Stx1, Stx2 , E. coli 72, E. coli O157:H7
samples by PCR Genotyping to detect the virulence factors (Stx1 and Stx2) of E.coli O157 :H7 isolates .

Materials And Methods:
A total of 80 locally produced cow's and buffalo's soft cheese samples (250 gm each) were collected randomly at weekly intervals (5 samples /week) in a sterile 500ml polyethylen plastic bags from various retail markets in different locations of Babylon province during two climatic periods (20 samples /species/ season), where the first period was in winter that extended from the beginning of December 2015 to the end of February 2016 while the second period was in summer that extended from the beginning of July to the end of September 2016. All the collected cheese samples (250 gm each) were transported to the laboratory of veterinary public health department at the college of veterinary medicine inside a portable ice-cooled box. All the microbiological tests were performed on arrival of samples in order to isolate and identify the E.coli O157:H7 from the samples.

Rapid Latex test kit:
Latex agglutination test kit as Remel Wellcolex Diagnostic Kit was imported from Remel Europe Ltd clipper Boulevard wet, cross ways Dartford, Kent, UK.

Molecular Detection of verotoxine genes (Stx1 and Stx2) by using PCR technique:
Using commercially available DNA extraction and Genomic DNA of E.coli O157:H7 isolates was extracted by using (Wizard Genomic ™ Mini g DNA Bacteria Kit Promega,USA) as in the purification kit (Geneaid, USA). The purified DNA was detected by electrophoresis in 1.5% agarose gel with addition of ethidium bromide. Bromophenol blue stain added to the DNA sample and visualizes the DNA by U.V. light.

Stx1: f
ATAAATCGCCATTGGAGACTAC
Stx1: r
AGAAGCCCGGTGGAGATCC

Stx2: f
GGCAGCTGCTGAAACTGCTCC
Stx2: r
TCGCCAGTTATCTGACATTCTG.
Protocol of Pollared et al., (1990) was used to study the Stx1 genes. This was done by using customize primers shown above. The PCR reaction mixture contained 5 μl of green master mix, 5 μl of purified bacterial DNA, 2 μl of each forward and reverse primers, then the volume was completed to 25 μl by deionized water. The PCR tubes were transferred to the thermalcycler (after centrifuged for 10 seconds) to start the amplification reaction according to the specific program for each gene. The results of the PCR were performed in post amplification process. 10 μl from amplified sample was directly loaded in a 1.5% 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 100 V. for 1 hr, then the products were visualized by UV transilluminator (Sambrook et al., 1989).

The data was analyzed by using the statistical analysis system named SAS (2010) and the significant differences were calculated with (p<0.05). The statistical analysis of the results were performed by F test (one way ANOVA), the least significant differences (LSD) were made to show the significant differences between the averages.

Results:
This study includes detection of the Verotoxin- producing Escherichia coli O157:H7 (VTEC O157:H7) in collected samples from locally made soft cheese after isolation on selective media and identification by biochemical tests, latex agglutination test, and by duplex PCR technique. The results of confirming the diagnosis of E.coli O157:H7 by PCR
assay are show in Figure 1 and Table 1 & 2. Six isolates of culturing serotype *E. coli* O157:H7 were positive by PCR assay where five of these isolates expressed gene of Stx1 whereas one isolate expressed gene of Stx2.

**Table (1): The prevalence level of *E. coli* O157:H7 in bovine soft cheese samples.**

<table>
<thead>
<tr>
<th>Source of cheese</th>
<th>Number of samples examined</th>
<th>Number of culturing positive samples</th>
<th>+ve PCR</th>
<th>% of <em>E. coli</em> O157:H7 isolates</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cows</td>
<td>20</td>
<td>10</td>
<td>4</td>
<td>40</td>
</tr>
<tr>
<td>Buffaloes</td>
<td>20</td>
<td>8</td>
<td>2</td>
<td>25</td>
</tr>
<tr>
<td>Total</td>
<td>40</td>
<td>18</td>
<td>6</td>
<td>33.33</td>
</tr>
</tbody>
</table>

**Table (2): Distribution Stx1 and Stx2 genes in *E. coli* O157:H7 isolates from soft cheese samples.**

<table>
<thead>
<tr>
<th>No. Of isolates</th>
<th>+ ve culturing</th>
<th>+ ve PCR</th>
<th>Stx1</th>
<th>Stx2</th>
</tr>
</thead>
<tbody>
<tr>
<td>6</td>
<td>6</td>
<td>6</td>
<td>5</td>
<td>1</td>
</tr>
</tbody>
</table>
The seasonal variation in the prevalence of *E. coli* O157:H7 in the locally produced bovine soft cheese samples collected from different local retail markets in Babylon province are shown in Tables 3, 4 and 5. The results established the statistically significant (p<0.05) influence of the season on the prevalence of *E. coli* O157:H7 in the bovine soft cheese samples. Data revealed that there was a significant (p<0.05) seasonal variation in the prevalence of *E. coli* O157:H7 where all the cow's and Buffalo's soft cheese samples had significantly (p<0.05) higher prevalence of *E. coli* O157:H7 in summer season (50% and 40% respectively) than in winter season (25% and 15% respectively). It was also found that all of the 40 cows and Buffalos soft cheese samples that were collected for each season had significantly (p<0.05) higher prevalence of *E. coli* O157:H7 in summer season (45%) than in winter season (20%).

**Table (3): The prevalence of *E. coli* O157:H7 in locally produced bovine soft cheese samples collected from Babylon province during the summer season.**

<table>
<thead>
<tr>
<th>Source of cheese</th>
<th>Number of samples examined</th>
<th>Number of positive samples</th>
<th>Percentage of positive isolating samples</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cows</td>
<td>20</td>
<td>10</td>
<td>50%</td>
</tr>
<tr>
<td>Buffaloes</td>
<td>20</td>
<td>8</td>
<td>40%</td>
</tr>
</tbody>
</table>
Table (4): The prevalence of *E. coli O157:H7* in locally produced bovine soft cheese samples collected from Babylon province during the winter season.

<table>
<thead>
<tr>
<th>Source of cheese</th>
<th>Number of samples examined</th>
<th>Number of positive samples</th>
<th>Percentage of positive samples</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cows</td>
<td>20</td>
<td>5</td>
<td>25%</td>
</tr>
<tr>
<td>Buffaloes</td>
<td>20</td>
<td>3</td>
<td>15%</td>
</tr>
<tr>
<td>Total</td>
<td>40</td>
<td>8</td>
<td>20%</td>
</tr>
</tbody>
</table>

Table (5): Seasonal variation in the prevalence of *E. coli O157:H7* in the locally produced bovine soft cheese samples collected from retail markets in Babylon province.

<table>
<thead>
<tr>
<th>Source of cheese</th>
<th>Number of samples examined per season</th>
<th>Percentage of positive samples</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>summer</td>
</tr>
<tr>
<td>Cows</td>
<td>20</td>
<td>50%</td>
</tr>
<tr>
<td>Buffaloes</td>
<td>20</td>
<td>40%</td>
</tr>
<tr>
<td>Total</td>
<td>40</td>
<td>45%</td>
</tr>
</tbody>
</table>

**Discussions**

The results of confirming the diagnosis of *E. coli O157:H7* by PCR assay are shown in Figure 1. Tables 1 & 2. Six culturing isolates serotype *E. coli* O157:H7 were positive by PCR assay where five of them expressed gene of Stx1 whereas one isolate expressed gene of Stx2. The present finding showed that 6 (33.33%) out of 18 positive soft cheese samples were positive for *E. coli O157:H7* isolates by PCR and these results indicated that the bovine served as an important natural source of humans infection by *E. coli O157:H7* through the contamination of soft cheese samples. However, EHEC colonization in adult ruminants was asymptomatic (Wray et al., 2000), it was hypothesized that the lack of stx receptors on enterocytes, was the cause of bovine asymptomatic infection by this organism (Kaper et al., 2004).

The result of study was in agreement with (Rey et al., 2003), who suggested that *E. coli O157* might be present in healthy domestic ruminants such as sheep, goats and they isolated...
this organism in rate 1% out of 697 healthy lambs in Spain, also in Egypt, Hiko et al., (2008), recorded that the prevalence of \( E.coli \ O157:H7 \) in raw meat products was 2.5% and 2% in sheep and goats respectively. Primers were used to detect \( E.coli \ O157:H7 \) at genus level and were prepared according to the informations of the company. Shah et al.,(2009) reported that PCR assay were proven as a specific and sensitive for detecting microbial pathogens such as \( E.coli \ O157:H7 \).Also Saeed and Ibrahim (2013) reported that gene based method such as PCR technique was more reliable than both the biochemical and the seriological tests for the diagnosis of \( E.coli \ O157:H7 \). The main advantage of the employed PCR techique was its ability to detect rough isolates or the isolates having a masked O antigen (Desmarchies et al.,1998).

The Table (3) and (4) indicated that the locally produced soft cheese were contamination by bacteria with \( Escherichia \ coli \) intestinal hemorrhagic (\( E.coli \ O157:H7 \)) higher in summer compared to in winter seasons, where the proportion of isolation during the summer was (45%) and in the winter was (20%), and this percentage was very high compared to the rates globally documented isolation had pointed out by (Mora et al. 2007) who isolated the bacteria in (8) out of 102 samples of bovine soft cheese in Peru (7.8%) and (7.6%) of soft cheeses made from raw cow's milk in Canada (Honish et al., 2005) have also been isolated from (4%) of soft cheeses in Turkey(Oksuz et al.2004). Dunn et al.,(2004). investigated that STEC were excreted at higher frequency in the warmer months and at lower frequency in cold months. Cases of \( E.coli \ O157: H7 \) outbreaks in humans were seasonal with the majority occurring between June and September (Besser et al ,1999). Flies have also been found to carry \( E.coli \ O157: H7 \) and can be responsible for transmission on farms (Ahmed et al.,2007; Alam et al.,2004).Both the mastitic udder and the fecal contamination are regarded as the important routes for the \( E.coli \ O157: H7 \) to enter the milk supply (Lira et al.,1995). Contaminated ground with feces has been identified as the source of infection in 48 out of 196 \( E.coli \ O157: H7 \) outbreaks documented in USA between 1982 to 1998 (Meng et al., 2001).  

**Reference:**


and veterinary science (IOSR-JAVS),23319-2372.6(2):30-32.


