Immunomagnatic Separation of *E.coli* O157:H7 from Raw Milk and Locally Produced Soft Cheese in Baghdad City

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Summary

The main objectives of the present study were to assess the incidence of *E .coli O157:H7* serotype in both raw milk and locally produced soft cheese samples and to evaluate the efficacy of new immunomagnetic separation (IMS) technique for the first time in Iraq for isolation of the same serotype from such products compared to the conventional cultural method .A total of 50 raw milk and soft cheese samples (25 samples of each) were collected randomly at weekly intervals from different retail markets in Baghdad province and its surroundings during the period of six months (from October 2011 March 2012). Each sample was divided into two equal parts where the first part was analyzed for the conventional cultural method and the second part was analyzed for the immunomagnetic separation technique. Five isolates (20%) and seven (28%) were identified as *E.coli O157:*H7 from the same raw milk samples by both the conventional cultural method and (IMS) technique respectively. Two (8%) and four isolates (16%) were identified as *E.coli O157:*H7 the same soft cheese samples using the same above methods. The detection limits by the conventional cultural method were $4x10^3$ cfu/ml and $7x10^2$ cfu/gm of raw milk and soft cheese samples respectively, while the detection limits by the (IMS) technique were $1x10^2$ cfu/ml and 12x10 cfu/gm of raw milk and soft cheese sample respectively .Results obtained in this study revealed that the IMS technique has been recognized to be significantly ($P<0.05$) more efficient in its sensitivity for the detection of low numbers of *E.coli O157:*H7 than the direct plating conventional method for both raw milk and soft cheese samples.

Keywords: Immunomagnatic separation, *E.coli* , Milk, Soft Cheese.

Introduction

Dairy cattle are known to be the most important reservoirs of *E.coli O157:* H7and can potentially enter milk during milking process through fecal contamination(1).Raw milk has long been recognized as a vehicle for the transmission variety of microbial pathogens therefore potential contamination levels could be reduced by improving sanitation and hygiene during milking (2) .Raw milk and its dairy products were implicated in an increasing number of cases and outbreaks of *E.coli O157:* H7infection (3) .The majority of *E.coli O157: H7* human infections occur in the summer and early Autumn months (4)*E.coli O157: H7* has recently been recognized as a new emerging pathogen for man ,causes diarrhea , Haemorrhagic colitis (HC), Haemolytic uremic Syndrome (HUS) and thrombotic thrombocytopenic purpura (TTP) with a low infective dose (5). Najim e*t al.,* (6) reported that 11 out of 24 (45.84%) of soft cheese samples that were made from raw milk in Baghdad province has Enterohaemorrhagic *E. coli O157: H*7.A wide variety of methods are currently available to detect and isolate *E. coli O157: H7* and since such serotype is often found in low numbers in food ,sensitive enrichment culture methods are required for detection (7). This enrichment step can also be followed by immunomagnatic separation (IMS) with beads coated with O157 specific antibody prior to plating onto agar (8).The introduction of pre-enrichment followed by IMS significantly increased the sensitivity of detection rates of *E.coliO157: H7* (9). The main objectives of the present study are to assess the incidence of *E.coli O157:H7* serotype in locally produced soft cheese made (on farm) from raw milk and evaluation the efficacy of new immunomagnatic separation technique compared to the conventional cultural method.

Materials and Methods

A total 50 raw milk (cow) and soft cheese samples (25samples of each) were collected randomly at weekly intervals from different retail markets in Baghdad province and its surrounding during the period of six months (October 2011 to march 2012). Each sample was divided into two equal parts in sterile pouches and transported to the laboratory inside ice box. The first part of each sample was analyzed by the conventional cultural method while the second part was analyzed by the immunomagnetic separation technique.

Isolation of *E.coli O157:H7* from soft cheese samples by immunomagnatic separation method. Twelve grams portion from the surface and the core of each soft cheese sample (25 samples) were extracted aseptically and added to 99 ml of sterile pepton water versus aqueous 2% Sodium citrate (Dual - purpose medium) enrichment medium which pre warmed to 40°C (10 and 11) and homogenized for 5 min in a stomacher.The homogenates were incubated at 37 °C for 24hrs, 1 ml of pre-enriched sample was added to 20 µl of anti-*E. coli*O157:H7 (Dynal) in 1.5 ml micro-centrifuge tube, and the tubes were incubated for 30 min at room temperature with constant mixing. The tubes were placed in a magnetic separator rack (MPC-M; Dynal, Oslo, Norway), the paramagnetic bead-bacterium complex was washed by being resuspended in sterilized saline solution, incubated for 1 min at room temperature with constant mixing (washing process were repeat 3-5 times), and left for 3 min. Finally, the paramagnetic bead-bacterium complex was suspended in 50 µl sterilized saline solution and 50 µl of this suspension was plated on Cefixime Tellurite- Sorbitol McConkey Agar (CT- SMAC; Oxoid) and chromo agar (Invitrogen). Putative *E. coli* O157:H7 colonies were subjected to serological confirmation by slide agglutination with anti-O157 serum and *E. coli* H antiserum H7 before subjected to biochemical confirmation (12).

Isolation of *E.coli O157:H7* from soft cheese (25 samples) by conventional cultural methods: Conventional methods were based on cultural, serological, and biochemical properties of *E.coli O157 :H 7* (13) .

Results and Discussion

The prevalence of *E. coli* O157:H7 isolates in raw milk and soft cheese samples by using both the conventional and IMS methods are shown in (table, 1).

Table, 1: Prevalence of *E.coli* O157:H7 in raw milk and soft cheese samples by conventional and immunomagnetic separation (IMS) methods

method	Number and type of sample	No. of positive isolates	Isolation percentage %	Counting of*E. coli* O157:H7 Mean)(
Conventional	25 milk	5	20	$4X10^3$cfu/ ml
IMS	25 milk	7	28	$1X10^2$cfu/ ml
Conventional	25 soft cheese	2	8	$7X10^2$cfu/ gm
IMS	25 soft cheese	4	16	12X10cfu/ gm

X^2 Cal = 0.160; df_1= 1; X^2 tab = 5.64.

Results obtained in this study revealed that five isolates (20%) and seven isolates (28%) were identified as *E. coli* O157:H7 from the same 25 raw milk samples by both the conventional cultural method and IMS technique respectively. Two isolates (8%) and four isolates (16%) were identified as *E.coli* O157:H7 from the same 25 soft cheese samples by both the conventional cultural methods and IMS technique respectively. The detection limits by the conventional cultural method were $3x10^3$cfu/ml and $7x10^2$ cfu/gm of raw milk and soft cheese samples respectively, while the detection limits by the IMS technique were $1x10^2$cfu/ml and 12 x10 cfu/gm of raw milk and soft cheese samples respectively. Results obtained in this study revealed that the IMS technique has been recognized to be significantly ($P<0.05$) more efficient in its sensitivity for the detection of low number of *E.coli* O157:H7compared to the direct plating (conventional method) from both raw milk and soft cheese samples (1).

Colonies of *E. coli* O157:H7 that were isolated from both raw milk and soft cheese samples were confirmed based on biochemical

and both cultural and serological characteristics are shown in tables 2 and 3 respectively *E. coli* O157:H7 was negative for potassium cyanide (no growth) and for both sorbitol and cellobiose fermentation (table,2).

Table, 2: Biochemical properties of *E.coli O157 :H7* isolates:

Medium	Reaction
Trypton broth (Indol test)	positive
Potassium cyanide (KCN)	Negative (No growth)
Motility test medium	Motile
Cellobiose fermentation	Negative
Sorbitol fermentation	Negative

E.coli O157:H7 colonies on nutrient ager were appeared as white colonies and on EMB agar showed the occurrence of green-metallic sheen on the surface of the colonies .Typical colonies of *E.coli* O157:H7 appeared on selective enrichment TC-SMAC agar as colorless with gray smoky center while on chromo agar appeared as mauve in color. Presumptive *E.coli* O157:H7 isolates obtained were further tested serologically for the presence of both the O157 and H7 antigens using the commercial available latex agglutination kits or antisera (Table, 3).

Table, 3: Cultural and Serological characteristics of *E.coli* O157 :H7 :

Medium /test	*E.coli*O157:H7 Results	Positive control result and Descriptions	Negative result and Descriptions
EMB	Positive	Greenish with dark center	Pink
Nutrient agar	Positive	White colonies	Yellow
TC-SMAC	Positive	Colorless with gray center (Sorbitol not fermented)	Pink to red colonies (Sorbitol fermented)
Chromogar™ *O15:H7*	Positive	Mauve colonies	Blue colonies
O antiserum	Positive	Agglutination	No Agglutination
H antiserum	Positive	Agglutination	No Agglutination

The isolation stage of *E. coli* O157:H7 can be shortened by replacing a selective enrichment stage with non-growth related procedures. Super- paramagnetic particles, which are coated with antibodies against the target microorganisms to selectively isolate the organisms from a mixed population, are used for immunomagentic separation (IMS). IMS is analogues to selective cultural enrichment, whereby the growth of other bacteria is suppressed while the pathogen of interest is allowed to grow. The separation process consists of two fundamental steps , where in the first step the suspension containing target cells is mixed with immunomagentic particles for a period of on longer than 30 minutes and finally they are separated using an appropriate magnetic separator. In the second step the magnetic complex is washed repeatedly to remove unwanted contaminants and the target cells with attached magnetic particles can be used for further experiments (14). The detection limit for IMS with 1cfu/1-25 g of sample following enrichment for *Listeria monocytogenes* (15).

It is difficult to compare studies that did not use IMS to recent studies that have utilized such technique. Some authors suggested that the IMS procedure is four times as sensitive as direct plating (16). Furthermore, another study using IMS recognized a sevenfold increase in prevalence of positive sample compared to direct plating and estimated that the IMS method could detect one organism per gram of feces (17). The United State Department of Agriculture (USDA) culture protocol, using IMS was found to isolate *E.coli* O157:H7 from fecal sample more than fivefold greater than direct plating technique (18). Detecting low levels of *E.coli* O157:H7 is considered important because of the small infective dose required to cause human disease .The use of IMS has been reported to improve the sensitivity of *E.coli* O157:H7 detection in food, human and bovine feces (19). Theoretically, enrichment followed by IMS should be able to detect as little as a single organism present in the initial sample. In laboratory however, the type and number of background bacterial flora, the type of broth used, and the temperature and incubation time impact the sensitivity of detection (20).

In conclusion prevention of undesired growth of microorganism (*E.coli* O157:H7) in raw milk and soft cheese could be achieved by improving sanitation and hygiene during milking and pasteurization of the milk

involves four steps: First; keep the microorganism out of milk or prevent its growth in milk (hygiene on the farm, quickly cooling the milk, short time between milking and cheese making). Second; kill the bacteria (Pasteurization). Third; manufacture the cheese to prevent contamination (dairy plant hygiene). The last step; create an environment within the cheese so that if the microorganism is present, its growth will be limited (proper pH, salt, fermentation of all sugar, low storage temperature). However the most universally accepted (but not always properly practiced) method of preventing defects by microorganisms is sanitation.

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فصل بكتريا الايشيريشيا القولونية المعوية النزفية O157: H7 بالحبيبات المغناطيسية من الحليب الخام والجبن الطري المصنع محليا في مدينة بغداد

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الخلاصة

الهدف الرئيس للدراسة الحالية هو تقييم وجود بكتريا الايشيريشيا القولونية المعوية النزفية *E.coliO157: H7* في كلا من عينات الحليب الخام والجبن الطري المنتج محليا وتقييم كفاءة تقنية العزل باستخدام الحبيبات المغناطيسية المغطاة بالاجسام المضادة وهي تقنية تستخدم للمرة الاولى في عزل السلالة نفسها من هذه المنتجات في العراق مقارنة بطريقة العزل التقليدية جمعت 50 عينة من الحليب الخام والجبن الطري (25 عينة من كلا النوعين) بشكل عشوائي اسبوعيا من اسواق البيع المختلفة في محافظة بغداد والمناطق المحيطة بها خلال مدة ستة اشهر (من بداية اكتوبر/ تشرين الاول 2011 حتى نهاية شهرمارس/اذار 2012). كل عينة قسمت الى جزءين متساوئين فحص الجزء الاول بالطريقه التقليدية والجزء الثاني بتقنية الفصل باستخدام الحبيبات المغناطيسية . شخصت خمسة عزلات (20%) وسبعة عزلات (28%) كونها *E.coliO157: H7* من عينات الحليب الخام بكلتا طريقتي العزل التقليدية وتقنية العزل باستخدام الحبيبات المغناطيسية وعلى التوالي . شخصت عزلتان (8%) واربعة عزلات (16 %)كونها *E.coliO157: H7* من عينات الجبن الطري والتي فحصت بكلتا طريقتي العزل التقليدية والفصل باستخدام الحبيبات المغناطيسية وعلى التوالي . كانت حدود الكشف بالطريقة التقليدية 4 X 10^3 وحدة تكوين المستعمرة/ مل و$7X10^2$وحدة تكوين المستعمرة /غم من عينات الحليب الخام والجبن الطري وعلى التوالي , بينما كانت حدود الكشف بطريقة الفصل باستخدام الحبيبات المغناطيسية 10^2X1 وحدة تكوين المستعمرة/مل و12 10Xوحدة تكوين المستعمرة/غم من عينات الحليب الخام والجبن الطري وعلى التوالي. اثبتت نتائج هذه الدراسة ان تقنية العزل باستخدام الحبيبات المغناطيسية هي الاكثر كفاءة وحساسية في الكشف عن الاعداد القليلة من هذه البكتريا بصورة معنوية وعلى مستوى ($P<0.05$) مقارنة بطريقة العزل التقليدية المباشرة من كلتا عينات الحليب الخام والجبن الطري.

الكلمات المفتاحية: الفصل بتقنية الحبيبات المغناطيسية , الايشيريشيا القولونية ,الحليب الخام , الجبن الطري.