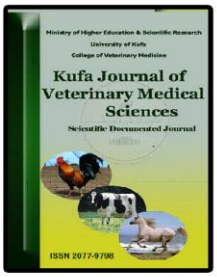
Kufa Journal for Veterinary Medical Sciences Vol.(8). No.(1) 2017

Kufa Journal for Veterinary Medical Sciences

vetmed@uoKufa.edu. iq



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

Najm Hadi Najim, Amer Jebur Obayes AL-Isawi

Dept.of Vet.Public Health
College of Veterinary Medicine, Baghdad University

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 periad was extended from the begining of December 2015 to the end of February 2016 while the second period was extended from the begining of July to the end of September 2016.The identification of *E.Coli O157:H7* isolates were confirmed based on their cultural, biochemical and seralogical characteristics using the commercial latex agglutination test kit and by duplex PCR technique. Data revealed that there was a significant ($P \leq 0.05$) seasonal variation in the prevalence of *E.Coli O157:H7* where all Cow's and Buffalo's soft cheese samples had significantly ($P \leq 0.05$) 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 postive by PCR assay and five of these isolates expressed gene of Stx1 whearse one isolate expressed gene of Stx2 .

KEYWORDS: **E. coli, PCR, Stx1 and Stx2 genes, Babylon.**

التحري الجزيئي عن ذيفانات الاشريشية القولونية *E.Coli O157:H7* والمعزولة من عينات الجبن المحلي للابقار والجاموس في محافظة بابل بتقنية PCR

الخلاصة

عزلت الأشريشيا القولونية *E.Coli O157:H7* من 80 عينة من عينات جبن الأبقار والجاموس محلي الصنع والتي جمعت بشكل عشوائي لفترات أسبوعية (5 عينة/ أسبوع) من أسواق البيع في مناطق مختلفة من محافظة بابل خلال فصلين (40 عينه/لكل نوع /فصل) حيث أمتدت الفترة الاولى من شهر كانون الأول 2015 الى نهاية شباط 2016 بينما الفترة الثانية أمتدت من بداية شهر تموز الى نهاية شهر أيلول 2016 بالاضافة الى ذلك جمعت 50 عينة براز من الابقار من مختلف الحقول في محافظة بابل لغرض عزل الاشريشة القولونية *E.Coli O157:H7* .تم عزل وتشخيص الاشريشية القولونية *E.Coli O157:H7* أعتمادا على الزرع والخصائص الكيموحيوية والسيرولوجية باستعمال أختبار تلازن اللاتكس التجاري . تشير البيانات على وجود فرق معنوي($P \leq 0.05$) باختلاف الفصل في أنتشار الاشريشيا القولونية *E.Coli O157:H7* في كل عينات جبن الابقار والجاموس وكان الأنتشار عالي

للاشريشيا القولونية *E.Coli O157:H7* في فصل الصيف (50% و 40% على التوالي) مقارنة بفصل الشتاء (25% و 15% على التوالي). وجد بأن في جميع عينات الجبن الطري المحلي (80 عينة لها أنتشار عالي المعنوية من الاشريشيا القولونية *E.Coli O157:H7* خلال فصل الصيف 45% مقارنة بفصل الشتاء 20% , بالاضافة لذلك وجد ان جميع عينات البراز البقري لها أنتشار عالي المعنوية للاشريشيا القولونية خلال فصل الصيف 72 % مقارنة بفصل الشتاء 40% . تم التاكيد على تشخيص *E.Coli O157:H7* بتقنية PCR حيث أعطت نتيجة موجبة لخمسة عزلات والتي عبرت عن جين Stx1 بينما عزلة واحدة عبرت عن جين Stx2.

الكلمات المفتاحية:الاشريشيا القولونية *E.Coli O157:H* 7 ,جينات Stx1, Stx2, الجبن الطري ,بابل

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, entrohaemorrhagic *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 (Derzelle *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

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 diffrenet locations of Babylon province during two climatic periods (20 samples /species/ season),where the first period was in winter that extended from the begining of December 2015 to the end of February 2016 while the second period was in summer that extended from the begining 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 protable 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 ethedium bromide. Bromophenol blue stain added to the DNA sample and visualizes the DNA by U.V. light.

Stx1:f ATAAATCGCCATTCGTTGACTAC.Stx1:rAGAACGCCCACTGAGATCATC

Stx2:f GGCACTGTCTGAAACTGCTCC.Stx2:rTCGCCAGTTATCTGACATTCTG.

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 postive by PCR assay where five of these isolates expressed gene of Stx1 whearse one isolate expressed gene of Stx2.

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

Source of cheese	Number of sampels examined	Number of cultural postive sampels	+ve PCR	% of *E.coli* O157:H7 isolates
Cows	20	10	4	40
Buffaloes	20	8	2	25
total	40	18	6	33.33

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

No. Of isolates	+ ve culturing	+ ve PCR	Stx1	Stx2
6	6	6	5	1

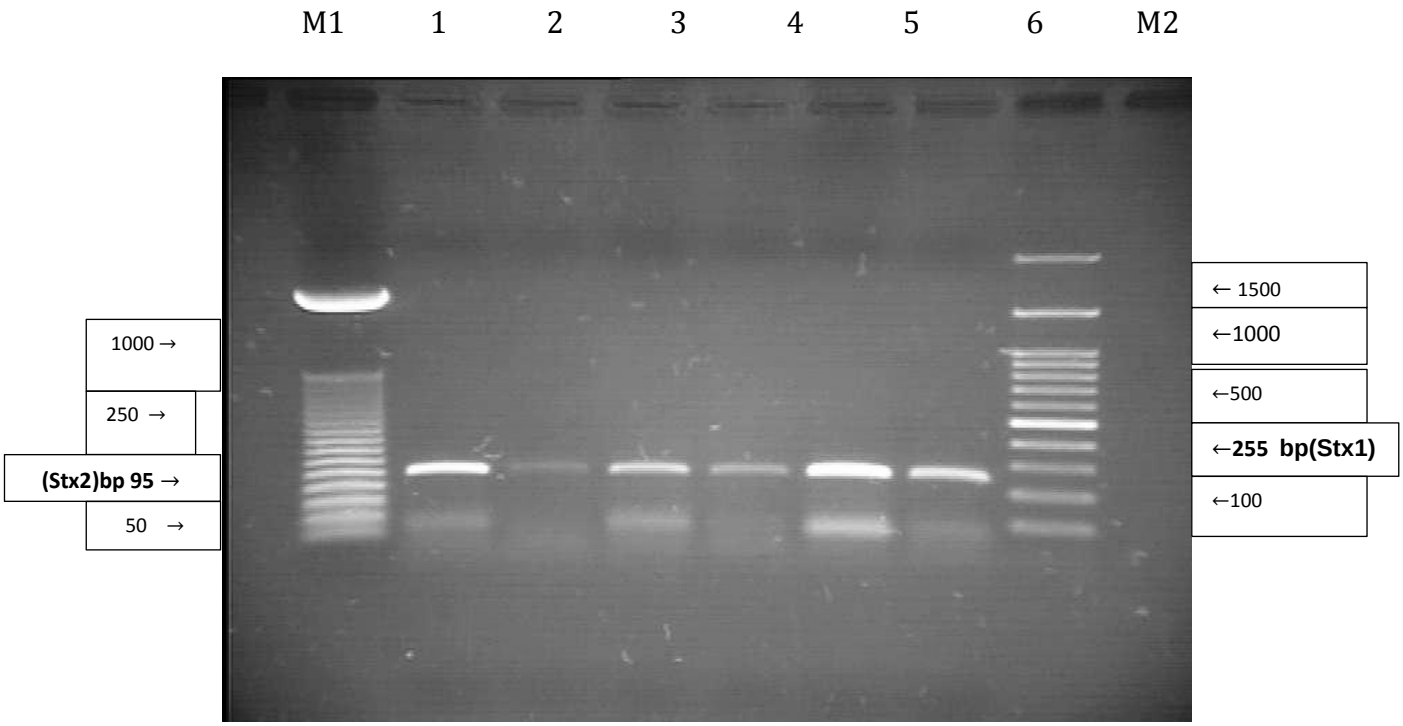


Figure 1: PCR amplification of Stx1 gene (255bp) lane 1,3,4,5,6 (postive result).lane M2 (100 bp lader).and amplification of Stx2 gene (95bp) land 5 (postive result).lane M1(50bp lader).

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.

Source of cheese	Number of sampels examined	Number of postive sampels	Percentage of positive isolating samples
Cows	20	10	50%
Buffaloes	20	8	40%

total	40	18	45%

Table(4):The prevalence of *E.coli O157:H7* in locally produced bovine soft cheese samples collected from Babylon province during the winter season.

Source of cheese	Number of sampels examined	Number of postive sampels	Percentage of postive samples
Cows	20	5	25%
Buffaloes	20	3	15%
total	40	8	20%

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.

Source of cheese	Number of samples examined per season	Percentage of positive samples	
		summer	winter
Cows	20	50%	25%
Buffaloes	20	40%	15%
Total	40	45%	20%

Discussions

The results of confirming the diagnosis of *E.coliO157:H7* by PCR assay are shown in in Figure 1 .Tables 1 & 2. Six culturing isolates serotype *E.coli* O157:H7 were postive by PCR assay where five of them expressed gene of Stx1 whearse 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.coliO157: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 aggrement 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 Spaine ,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 technique 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 routs for the *E.coli O157: H7 to* enter the milk supply (Lira et al.,1995). Contaminated ground with feces has been identificd 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:

Ahmad, A.; Nagaraja,T. G.;and Zurek, L.(2007). Transmission of *Escherichia coli* O157 : H7 to cattle by house flies. *Preventive Veterinary Medicine*, 80 (1): 74-81.

Alam,M.J.and Zurek,L.,(2004).Association of *Escherichia coli O157:H7* with houseflies on a cattle farm. *Applied and Environmental Microbiology*.70 (12):7578-7580.

Allerberger,F.;Kreidl,P.;Dierich,M.P;Klingsbichel,E.Jenewein,D.;Mader,C.Khaschabi,D.;Schonbauer.M.andC.Berghold.(2003).*Salmonella enterica* serotype Oranienburg infections associated with consumption of locally produced tyrolean cheese. *Euro Surveill.* 5:123-126.

Barletta, F.;Ochoa,T.J.;Ecker,L.;Gil,A.I.;Lanata, C.F.and Cleary,T.G.(2009). Validation of five-colony pool analysis using multiplex real-time PCR for detection of diarrheagenic Escherichia coli. J. Clin. Microbiol., 47:1915–1917.

Besser, R.E.; Griffin,P.M. and.Slutsker,L.,(1999). *Escherichia coli*O157:H7.

Derzelle,S.;Grine,A.;Madic,J.;de Garam, C.P.;Vingadassalon, N.; Dilasser, F.; Jamet, E.and.Auvray,F.(2011)Aquantitative PCR assay for the detection and quantification of Shiga toxin-producing *Escherichia coli* (STEC) in minced beef and dairy products.

International Journal of Food Microbiology. 151: 44-51.

Desmarchier,P.M.;Bilge,S.S.;Fegan,N.;Mills, L.;Vary,J.C.and Tarr,P.I.(1998).A PCR specific for *E coli* O157:H7 based on rfb lous encoding O157:H7 lipopoly saccharide.J.Clin.microbial., 36:1801-1804.

Dunn,J.R.;Keen,J.E. and Thompson,R.A.(2004).Prevalence of Shiga-toxigenic *Escherichia coli O157:H7* in adult dairy cattle.J.Am.Vet.Med. Assoc.,224:1151–1158.

Fratamico, P.M. and Smith, J.L.(2006). *Escherichia coli* infections.In Riemann, HP and Cliver,DO (Eds.),Foodborne infections and intoxications.(3rd Edn.),Florida, Academic Press, an imprint of Elsevier. PP: 205-208.

Govaris,A;Koidis,P.and Papatheodorou,K. (2001). The fate of Escherichia coli O157:H7 in Myzithra, Anthotyros, and Manouri whey cheeses during storage at 2 and 12°C. Food Microbiol.,18: 565-570.

Hiko, A. ; Asrat, D. and Zewde, G.(2008)**.**Occurrence of *E.coli O157:H7* in retail raw meat products in Ethiopia. J.Infect.Pevctries., 2:389-393.

Honish,L.;Predy,G.;Hislop,N.;Chui,L.;Kowal ewska,;Grochowska,K.;Tottier,L.;Krep linc and Zazulak,I.(2005).An outbreak of *Escherichia coli O157:H7* hemorrhagic colitis associated with unpasteurized gouda cheese ,clin. public health .96:(3) 182-4.

Kaper,J.B.;Nataro, J. P. and Mobley, H. L.,(2004).Pathogenic *Escherichia coli*. *Nature Reviews Microbiology*. 2 (2): 123-140.

Leyer, G.J.;Wang, L.L.and Johnson, E.A.(1995). Acid adaptation of Escherichia coli O157:H7 increases survival in acidic foods. Appl.Environ. Microbiol., 61: 3752-3755.

Maher, M. M.;Jordan, K.N.; Upton, M.E. and Coffey. A. (2001) Growth and survival of *E. coli* O157:H7 during the manufacture and ripening of a smear-ripened cheese produced from raw milk. *J. Appl. Microbiol.*, 90:201-207.

Meng,J.;Doyle,M.P.;Zhao, T. and Zhao.S.(2001). Enterohaemorragic *Escherichia coli*. In Food microbiology:fundamentals and frontiers. American Society of Microbiology Press, Washington, DC.

Mora,A.;Leon,S.L.;Blance,M.;Blance,J.E. Lopez ,C.and Dahbi,G.(2007).*Escherichia coli* Blanco ,J.(2007).Phage types virulence genes and (PFGF) Profiks of shiga toxin – production *Escherichia coli* O157:H7 isolated from raw beef soft cheese and vegetables in perv .Int . J. Food Microbiol .10 :114(2):204-10.

OKsus ,ő, ; Arici ,M.; Kurdtays, S. And Gümüs,T.(2004). Incidence of *Escherichia coli O157* in Raw milk and white pickled cheese manufactured from raw milk in Turkey . Food control. 15 (6): 453-456.

Pollard, D. R.; Johnson, W. M.; Lior, H.; Tyler, S. D. And Rozee, K. R.(1990).Rapid and specific detection of verotoxin genes in *Escherichia coli* by the polymerase chain reaction. J. Clinic. Microbiol. 28: 540-545.

Rendón, M.A.;Saldaña, Z.;Erdem, L.A.;Monteiro-Neto,V.;Vázquez,A. ;Kaper,B. J.; Puente, L.J.;Jorge A; and Girón,A. J.(2007)**.**"Commensal and pathogenic *Escherichia coli* use a common pilus adherence factor for epithelial cell colonization". PNAS. 104 :10637-10642.

Rey,J.;Blace,J.C.;Blaco,M.;Mora,A.;Dahbi,G. ;Alonso,J.M.and.Blaco,J.(2003).Serot pic,phagotypes and virulence genes of shigotoxic producing *E.coliO157* isolated from sheep in Spain.Vet.Microbiol.,94:47-56.

Saeed,A.Y.and Ibrahim,K.H.S.(2013).Identification of *E.coli O157:H7* in sheep and goats using PCR techingue.J.of Aqriculture

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

Sambrook, J.; Fritsch, E. F. and Maniatis. (1989). Molecular cloning 2nd ed. Cold spring Harbor Laboratory Press, N.Y. 1746-1753.

Shah,D.H.; Shringi, S.; Besser,T. E. and call, D. R.(2009).Escherichia.In Liu, D(Ed). Molecular detection of Foodborne pathogens,P369-389.Boca Raton ;CRC press Taylor and Francis group.

Vernozy-Rozand,C;Mazuy-Cruchaudet, C; Bavai, C; Montet, MP; Bonin, V; Dernburg, A and Richard,Y (2005). Growth and survival of Escherichia coli O157:H7 during the manufacture and ripening of raw goat milk lactic cheeses. Int.J.Food Microbial., 105: 83-88.

Wang, S. and Levin, R.E. (2006). Discrimination of viable *Vibrio vulnificus* cells from dead cells in real-time PCR.J.Microbial.Methods 64:1–8.

Wray,C. ;McLaren, I.M.and.Randall, L.P.(2000).Pearson GR.Natural and experimental infection of normal cattle with *Escherichia coli O157.* Vet Rec.,147:65–68.

Yoon,J.W.;and Hovde,C.J. (2008). All blood, nostool: enterohemorrhagi *Escherichia coli O157:H7* infection. J. Vet. Sci., 9: 219-231.