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Detection of *rfb_{O157}* and *$fliC_{H7}$* Genes in *Escherichia coli* Isolated from Human and Sheep in Basrah Province

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ABSTRACT

This study aimed to detect *Escherichia coli* O157:H7 in samples from patients with diarrhea, sheep milk, and sheep drinking water by growth on selective media, biochemical tests, latex agglutination test, and PCR technique.

During the period from November 2008 to May 2009, a total of 340 samples were collected; stools diarrhoeic patients (125), healthy sheep feces (125), milk (45), and sheep drinking water samples (45). Samples were screened for the presence of non-sorbitol fermenting colonies (NSF) on sorbitol MacConkey agar supplemented with cefixime and potassium tellurite (CT-SMAC). Out of the 340 samples, 58 (46.4%) stool from diarrhea, 98 (78.4%) from sheep feces, 19 (42.2%) milk, and 17 (37.7%) drinking water were non-sorbitol fermenting. *E. coli* isolates were serotyped as *E. coli* O157:H7 by latex agglutination test, 3 (5.7%) isolates of stool from diarrhea patients, 7 (7.3%) isolates of sheep feces, 2 (11.1%) isolates of milk, and none of drinking water samples were positive. All latex agglutination positive isolates were positive to PCR test except one isolate from sheep feces. PCR positive isolates were produced enterohemolysin on sheep blood agar, and were found resistant to at least 3 antibiotics.

The highest rate of *E. coli* O157:H7 isolation was observed in April (7.6%) followed by December (4.7%) , while those of sheep feces showed the highest rate in March (10%) followed by April (8.6%).

Keywords : *E. coli* O157:H7, *rfbO157* gene, *fliCH7* gene, PCR.

fliC$_{H7}$* *rfbo157

O157:H7

) 340 .PCR

(N=45) (N=125) (N=125)

.2009 / – 2008 / (N=45)

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(% 78.4) 98 (% 46.4) 58 340

. (% 37.7) 17 (42.2) 19

O157 : H7

(% 11.1) 2 (% 7.3) 7 (% 5.7) 3

.O157 :H7

PCR . PCR

.

O157:H7 .

(% 4.7) (% 7 .6)

.(% 8.6) (% 10)

fliCH7 *rfb O157* O157:H7 :

INTRODUCTION

Escherichia coli O157:H7 is a normal microflora of digestive tract in animals . Various subtypes of the bacterium can cause infections in humans (Belanger *et al*., 2002). One of these infections is caused by Shiga toxin producing *E. coli* (STEC), formerly known as verotoxin-producing *E. coli* (Eliot *et al*., 2001), which comprise several serotypes. In recent years one particular serotype has been considered pathogenic and named *E. coli* O157: H7.

Since its first recognition, this serotype and its isolation from stool samples have sharply increased , and ranked as the 3rd most common bacterial pathogen of the human gut after *Salmonella* and *Campylobacter* spp. (Allerberger *et al*., 1996 ; Adwan *et al*., 2002). Isolation of this bacterium from cases of diarrhea varies, although victims are mostly children under 5 years and occurrences are frequently in spring and summer seasons (Keskimaki *et al*., 1998 ; Klein *et al*., 2002).

E. coli O157:H7 causes a severe disease in humans that starts with profuse diarrhea, and if not diagnosed or treated properly can lead to serious complications such as hemorrhagic colitis, hemolytic uremic syndrome and thrombocytopenic purpura (Paton and Paton, 1998 ; Besser *et al*., 1999). Nevertheless, 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*., 1996). Unfortunately, the diagnosis of *E. coli* O157: H7, in spite of its role in diarrhea and its potentially severe outcome, is not considered a routine laboratory test in the third world countries, and except for very few reports, there are no comprehensive and documented data on the incidence and prevalence of diarrhea caused by this particular strain (Aslani *et al*., 1998; Fazeli *et al*., 2003). Thus this study was designed to detect *E. coli* O157:H7 in sheep as a major reservoir and as a causative agent for diarrhea patients, and to characterize these isolates by means of biochemical tests, serotyping and PCR techniques.

MATERIALS AND METHODS

Sample collection:

A total 340 samples (125 diarrhea patients stool samples, 125 sheep feces samples, 45 sheep milk samples and 45 sheep drinking water samples) were collected in the period from November 2008 to April 2009.

Isolation of *E. coli* O157:H7

Loop full of each stools and feces samples were enriched in Tryptic Soy Broth (TSB) supplemented with Vancomysin (4 mg/L) according to (Sanderson *et al*., 1995) and incubated in 37± 1°C for 24h. A loop full from each broth was streaked on Sorbitol – MaCconkey agar (Himedia) plates supplemented with Cefixime (0.05 mg/L) and potassium tellurite (2.5 mg/L) according to (Zadik *et al*., 1993). Plates were incubated at 37± 1 °C for 24h. Five non- sorbitol fermenting colonies were transferred by sterile tooth pick sticks to MaCconkey agar plates and incubated for further 24h at 37± 1 °C.

Biochemical tests (IMVIC, TSI and Cellobiose) were performed on the non-sorbitol fermenting colonies. Latex agglutination kit (Wellcolex *E. coli* O157:H7, Remel) was used for conformity identification of *E. coli* O157:H7 to detect the somatic antigen O157 and flagellar antigen H7.

Bacterial genomic DNA extraction and purification :

This procedure was done by using commercially available DNA extraction and purification kit (promega, USA).

Detection of the Whole DNA

The purified DNA was detected by electrophoresis (60 V) in 1% agarose gel with ethedium bromide. Methelen blue stain added to the DNA sample and visualizes the DNA by the UV light.

Amplification of *rfb*$_{O157}$ and *flic* $_{H7}$ genes:

rfb$_{O157}$ and *flic* $_{H7}$ are genes encoding for the O157 somatic and H7 flagellar antigens respectively. This was estimated by using customized primers (Table 1). The amplification mixture (Alpha, Canada) contain 12.5 µl of green master mix (*Taq* DNA polymerase, dNTPs, $MgCl_2$ and reaction buffer at optimal concentration for efficient amplification of DNA templates by PCR), 5 µl of purified DNA , 2.5 µl of each forward and reverse primers, then volume completed to 25 µl by deionized water. All tubes were centrifuged in microcentrifuge for 10 seconds. PCR tubes were transferred to the thermalcycler to start the amplification reaction according to a specific program for each gene (Gannon *et al*., 1997 ; Paton and Paton, 1998). Agarose gel was prepared in two concentrations (2% and 1.5% for *rfb*$_{O157}$ and *fliC*$_{H7}$ respectively), with voltage supply set in 75 volt for 35-45 min. .

Table 1: Oligonucleotide primers sequences used for PCR amplification.

Primer	Sequence (5'-3')	Amplicon size (bp)	Reference
rfb$_{O157}$	F: CGGACATCCATGTGATATGG R: TTGCCTATGTACAGCTAATCC	259	Paton and Paton (1998a)
fliC$_{H7}$	F: GCGCTGTCGAGTTCTATCGAGC R: CAACGGTGACTTTATCGCCATTCC	625	Gannon *et al*. (1997)

Enterohemolysin production test:

Fresh colonies from the PCR positive isolates were streaked on sheep blood agar plates and incubated at 37± 1 °C for 24 h. .The plates which exhibited hemolysis (α hemolysis) after 8h were excluded. The target bacteria develop small, clear zones of β- hemolysis around the colonies after 24h (Schmidt *et al*., 1994).

Antibiotic Susceptibility test:

Antibiotic susceptibility test was performed by disc diffusion method to measure the patterns antibiotics resistance among the isolates (Bauer *et al*., 1996). Nine antibiotics (Bioanalyse) were used. The Muller- Hinton agar plates were swabbed with bacterial suspension by sterile cotton swabs. Using sterile forceps, the antibiotic discs were placed on the plates. The plates were incubated at 37± 1°C for 24 h, then the diameter of inhibition zones were measured and compared with a standard table (Bioanalyse / Turkey).

RESULTS

Frequency of non sorbitol fermenting (NSF) *E.coli*

The frequency of NSF *E. coli* was 46.4% in human stool samples, 78.4% in sheep fecal samples, 42.2% in milk samples, and 37.7% in water samples based on non-sorbitol fermintation in TC-SMAC. Based on Chi-squar statistical analysis, the results revealed significant differences between the isolation frequency from human and sheep while there are no significant differences in isolation frequency in human, milk, and water samples (Table 2).

Table 2: Frequency of NSF *E. coli* in human, sheep, milk, and water.

Isolates source	Total samples	Non sorbitol fermenting	%
Human	125	58	46.4
Sheep	125	98	78.4
Milk	45	19	42.2
Water	45	17	37.7
Total	340	192	56.5
X^2 = 39.725		**$p<0.01$**	

Biochemical tests for *E. coli*

Biochemical identification tests (IMVIC, TSI, and Cellobiose) of NSF colonies showed that 89.6%, 96.9%, 94.7%, and 76.4% of human, sheep, milk, and water isolates were positive *E. coli* respectively. A statistical analysis showed a significant difference between the isolation frequencies.

Serological identification of *E. coli* O157:H7

Quality detection of somatic O157 antigen was done by latex agglutination test . The results revealed that 11.5%, 11.5%, 16.6%, and 23% of *E. coli* isolates from human, sheep, milk, and water respectively were positive for O157 somatic antigen. The O157 positive isolates tested for the flagellar H7 antigen. Frequency of H7 antigen positive isolates were 5.7%, 7.3%, 11.1%, and 0% for human, sheep, milk, and water isolates respectively (Table 3). Statistical analysis revealed no significant differences in the frequency of *E. coli* O157:H7 from different sources.

Table 3: Frequency of latex agglutination positive *E. coli* O157:H7

Isolates source	*E. coli* isolates	O157 positive	%	H7 positive	%
Human	52	6	11.5	3	5.7
Sheep	95	11	11.5	7	7.3
Milk	18	3	16.6	2	11.1
Water	13	3	23	0	0
X^2=0.979 p>0.05			**X^2=1.975 p>0.05**		

Detection of *rfb_{O157}* and *$fliC_{H7}$* genes

The rfb_{O157} gene size (259 bp) was estimated depending on DNA marker (100 bp DNA ladder). There were 11.5%, 11.5%, 16.6%, and 23% positive isolates from human, sheep, milk, and water samples respectively (Table 4, Fig. 1). The rfb_{O157} positive isolates then tested for the presence of $fliC_{H7}$ gene (625 bp). The results were 5.7% , 6.3% , 11.1% , and 0% positive isolates from human, sheep, milk, and water samples respectively (Table 4, Fig. 2). There were no significant differences in the frequency of rfb_{O157} and $fliC_{\mathrm{H7}}$ in the isolates from different sources.

Table 4: Frequency of rfb_{O157} and $fliC_{H7}$ in the samples.

Isolates source	Total	*E. coli* isolates	rfb_{O157} gene	%	$fliC_{\mathrm{H7}}$ gene	%
Human	125	52	6	11.5	3	5.7
Sheep	125	95	11	11.5	6	6.3
Milk	45	18	3	16.6	2	11.1
Water	45	13	3	23	0	0
X^2 = 1.904				**P>0.05**		

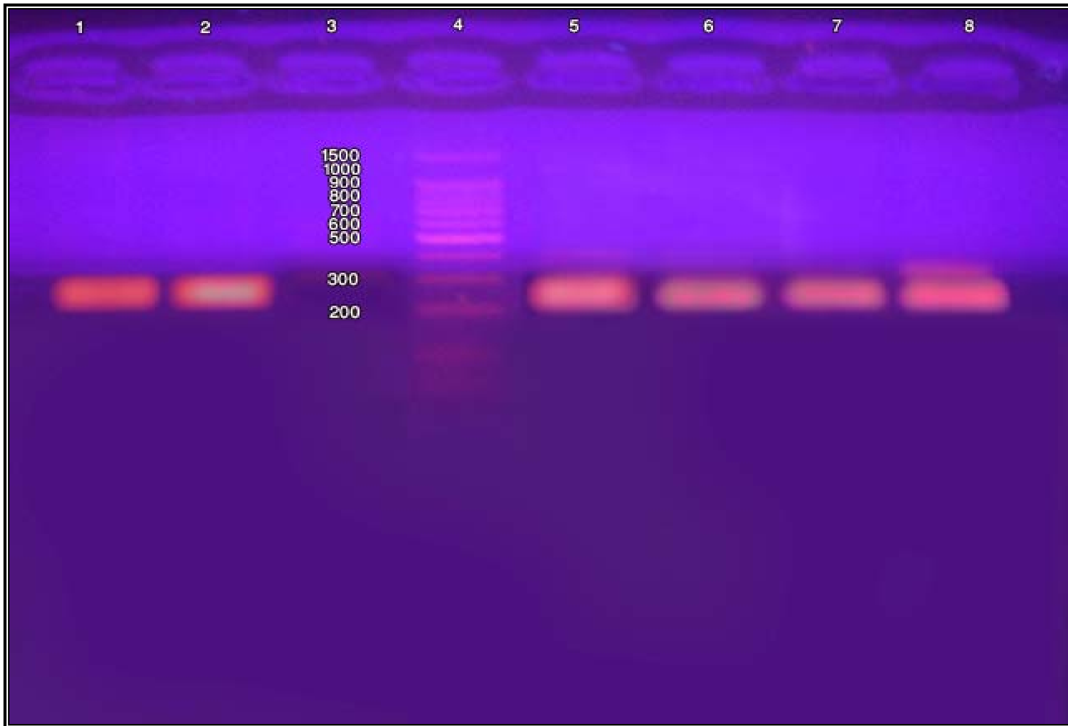

Fig. 1: PCR amplification of *rfb*$_{O157}$ gene (259 bp) Lane 1, 2, 5 – 8; lane 4 ladder.

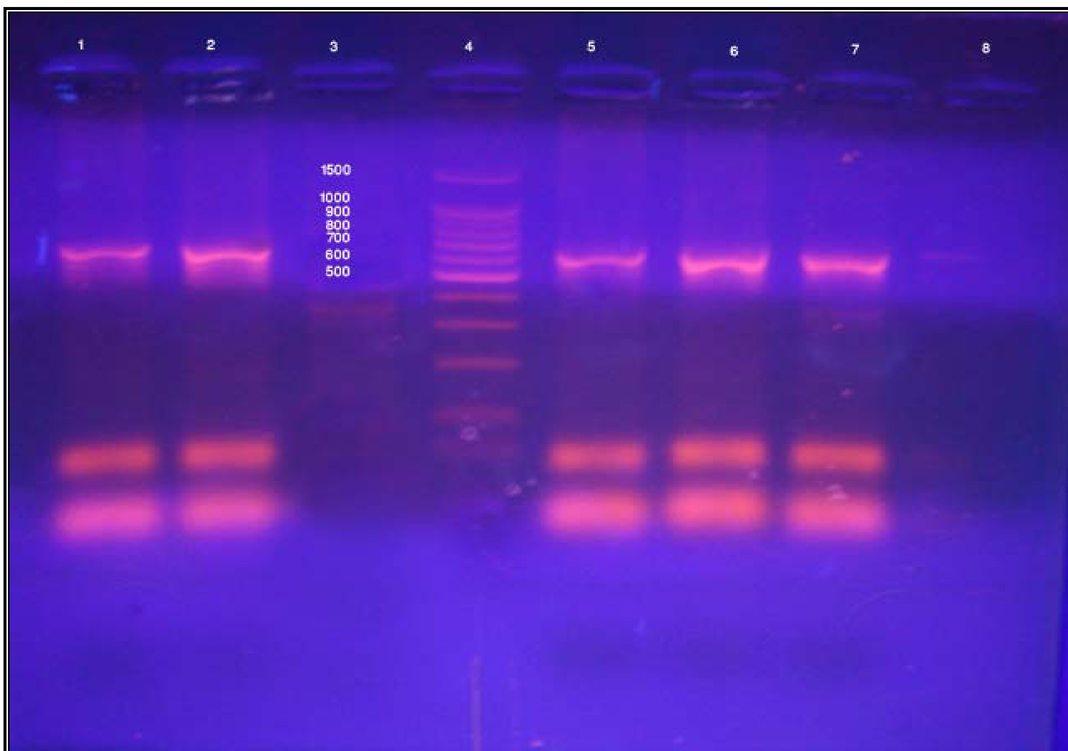

Fig. 2: PCR amplification of *fliC*$_{H7}$ gene (625 bp) lane 1, 2, 5 – 8; lane 4 ladder.

Detection of Enterohemolysin Production

All the *E. coli* O157:H7 isolates (11 isolates) were tested for the production of enterohaemolysis on sheep blood agar (SBA). All tested isolates were positive β haemolysis and have enterohaemolysin activity.

DISCUSSION

In the present study, conventional culture method was performed on TC – SMAC agar. The frequency of NSF isolates in human stool samples was 46% and 78.4 for sheep fecal samples. These results reflect the importance role of EHEC as causative agent of diarrhea; also emphasize the role of sheep as major reservoir for pathogenic *E. coli*. The frequency of NSF isolates in human fecal samples 46% was higher than the rate reported by (Issa, 1997) who reported that NSF isolates from inpatient children was 28.57%, also higher than (Naaham, 2004) who showed isolation rate 27.3%. Other Iraqi studies such as (Shebib, 2000) who reported lower NSF isolates rate (5% , 1.14% respectively) compared with the rate in the present study. The differences in isolation rate between the present study and the other studies may be related to the selective enrichment procedure used in this study which revives the stressed and injured cells. On the other hand, the selective medium (TC-SMAC) in this study inhibits other enteric organisms to compete with and over grow the targeted organism of the present study.

The rate of NSF isolates from sheep fecal samples (78.4%) was higher than the rate (71.25%, 22.2%) reported by (Kudva *et al*., 1996 ; Djordjevic *et al*., 2001). There are many reasons that can explain the high rate in the present study, first in the present study most sheep were raised in mutual habitat with cattle which consider major reservoir of pathogenic *E. coli*. Also tested sheep were grazed on garbage and insanitary feedlot bins which increase the contamination rate. On other hand, genetic variations between sheep breed in the present study (local breed) and the previous studies (crossed meat breed and Merino respectively).

Latex agglutination test is sensitive and specific in identification of *E. coli* O157:H7. Also it is simple and easy to use. The use of this method in the present study was to reduce the time and effort use in isolation, also to eliminate other serotypes of pathogenic *E. coli* which have the cultural and biochemical character. In the present study, the rate of positive isolates to *E. coli* O157:H7 latex agglutination test were 5.7% , 7.3% , 11.1% , and 0% for samples of human, sheep, milk, and water respectively. The rate of recovery of *E. coli* O157:H7 from diarrhea patients in the present study positive to latex agglutination test (5.1%) was higher than that recovered by (Aslani and Bouzari., 2003 ; Allerberger *et al*., 1996 ; Klein *et al*., 2002) which were 1.4% , 1% , and 1.5% respectively. The high rate of recovery in the present study can be explained by the area from which patients came from, that 98% of them came from rural areas and families of animals owners. The direct contact with animals, material contaminated with animal feces or contaminated buildings where animal are confined, this will increase the chances of infection.

A CDC report in 2005 about outbreak in North Carolina with *E. coli* O157:H7 refer that 78% of them reported visiting petting zoo at the state fair. On other hand, the lack of insanitary conditions in rural areas was important reason. The present finding was concordant with the above studies, whereas other researchers have reported away higher rates; for example in Lagos, Nigeria, (Olorunshola *et al*., 2000) recovered *E. coli* O157:H7 from 6% of diarrhea patients. (Adwan *et al*., 2002) during an outbreak of diarrhea in

northern Palestine examined 250 stool samples and reported 124 positive cases of *E. coli* O157:H7 (about 50%). In the latter study, the reasons that can be suggested for this high frequency of recovery of the serotype are the timing of sample collection since it was during an outbreak of diarrhea and overcrowding, insanitary conditions and poverty in the area.

In the present study, rate of *E. coli* O157:H7 positive isolates for latex agglutination test from sheep was (7.1%). This rate is higher than that obtained by (Djordjevic *et al.* , 2001; Blanco *et al*., 2003; Chapman *et al*., 1997) 2.2% , 1% and 2% respectively. This difference in isolation rate can returned to the geographical differences in as previous studies had conducted in another continent (Europe) except Djordjevic *et al*.,(2001) (Australia) in which other serotypes such as O91:H- , O5:H- , O128:H2 and O85:H49 and many other were most common isolated respectively (Djordjevic *et al*., 2001 ; Sidjabat-Tambunan *et al*., 1997). In contrast, Long *et al*. (2003) and Novotna *et al*.(2005) who isolated *E. coli* O157:H7 in much higher rate (40%) and (25%) respectively than in the present study. On other hand, our result was concordant with result obtained by (Heuvelink *et al*., 1998) which was 4%.

The rate of milk positive isolates for latex agglutination test in the present study was 10.5% concordant with result obtained by (Wells *et al*., 1991 ; Abdul-Raouf *et al*., 1996) 4.3% and 6% respectively while it is higher than result obtained by (Clarke *et al*., 1989; Oksuz *et al*., 2003; Karns *et al*., 2007; Murinda *et al*., 2002) which were 1.9%, 1%, 0.02%, and 0.75% respectively. This difference in rate of isolation may return to the unhygienic ways of milk collection and the use of contaminated equipments by the animals' feces. Also the warm climate in most seasons in Basrah provides a perfect condition for the bacteria to survive and proliferate for long period. A study done by (Massa *et al*., 1999) show good survival or even multiplication of *E. coli* O157:H7 in raw milk when stored at 8°C. So milk should be stored at least in 5°C and that in fact was not done by local owners.

In the present study, no *E. coli* O157:H7 isolates obtained from water sample by latex agglutination test or PCR technique. In contrast, (LeJeune *et al*., 2001) reported isolation 1.3% of *E. coli* O157:H7 from water troughs in farms in Washington, Oregon, and Idaho, USA. This result can be returned to many reasons, first, in LeJeune *et al*. study all troughs which gave positive results for *E. coli* O157:H7 were located in covered areas with no exposure to sun light. Direct sun light lower the survival chances of *E. coli* in the aquatic system (Barcina *et al*., 1989). On other hand, it is common for bacteria to concentrate up to 1000 time higher in sediments than in the overlying water column (Ashbolt *et al*., 1993), thus difference in sampling method in our study (surface water) and the other study reflected in the isolation rate. Also in the present study the water was provided directly from the river to the animals, so the competition with other organisms already present in water influence the survival of *E. coli* in water (Gonzalez *et al*., 1992 ; Marino and Gannon,1991).

In recent years, PCR-based detection method becomes very important as a technique for the detection of bacteria. The main reason for this is the DNA from a single bacterial cell can be amplified in about 1 hour, which is very rapid compared to the methods described previously (Jaykus, 2003 ;Winfield and Groisman, 2003).

In the present study, frequency of isolates from human, sheep, milk, and water which confirmed positive as *E. coli* O157:H7 by detection of both rfb_{O157} and $fliC_{H7}$ genes was 5.7% , 7.3% , 11.1% and 0% respectively.

The rate of PCR positive for *E. coli* O157:H7 isolates from human samples (5.7%) was concordant with results obtained by (Gunzer *et al.*, 1992) which was 3%. On other hand, our rate of isolation was much higher from that (0.5%, 0.4%) obtained by (Blanco *et al.*, 2004). The high rate recorded in the present study can be returned to the use of primers designed to detect genes encoded for somatic O157 antigen and flagellar H7 antigen which give highly specific and sensitive that cannot be crossed reaction with other serotypes, while in the previously mentioned studies they use primers designed to target genes stx_1, stx_2, and *eae* which encoded for Stx1, Stx2 , and intimin respectively which may found in other serotypes than *E. coli* O157:H7. Blanco *et al.*, 2004 found that 43.6% of the non-O157 serotype carried at least 2 of 3 (stx_1, stx_2, and *eae*) genes been detected, so the use of these genes as indicator for *E. coli* O157:H7 may be not reliable.

The rate of PCR positive *E. coli* O157:H7 isolates from sheep samples (6.1%) was higher than that obtained by (Pao *et al.*, 2005; Heuvelink *et al.*, 1998, Blanco *et al.*, 2003; Chapman *et al.*, 1997 ; Djordjevic *et al.*, 2001) which were 1.4%, 4%, 0.4%, 2%, and 2.2% respectively. The differences in isolation rate between the recent study and the other mentioned studies may be related to dietary methods which used in feed of the animals (Rassmussen *et al.*, 1993), that all animals tested in this study were fed in free rang method. Moreover, most of tested sheep either live with cattle, graze with cattle or graze in areas contaminated with cattle's manure and as known cattle are the major reservoir of *E. coli* O157:H7. In contrast, in Norway (Johnson *et al.*, 2001) did not isolate *E. coli* O157:H7 from 364 tested sheep. In other hand, isolation rate from sheep corresponded with isolation rate from cattle (major reservoir) obtained by Chapman *et al.*(1997) which were 38%, Elder *et al.*(2000) (28%), Low *et al.*, (2005) (13%), and Islam *et al.*(2008) (7.2%). while other researchers (Laegreid *et al.*, 1999 ; Wells *et al.*, 1991 ; Garber *et al.*, 1995) had obtained rates (6.9%, 5.9%, and 5% respectively) similar to rate in the present study.

The antibiotic susceptibility test was performed on the positive *E.coli* O157:H7 from all sources (human, sheep, milk, and water). All isolates were found to be resistance to Cephalothin and Erythromycin, This result was concordant with (Fard *et al.*, 2008 ; Naahma, 2004). While in contrast, (Schroeder *et al.*, 2002) found only 15% of *E. coli* O157:H7 were resistant to Cephalothin. In other hand, all isolates were sensitive to Nalidixic acid, this result was in agreement with results obtained by (Feder *et al.*, 2003) but less sensitive to Ciprofloxacin, Trimethoprim, and Cefotaxime (81.8%, 72.7%, and 27.3% respectively) and this correspond with results obtained by (Ratnam *et al.*,1988 ; Fazeli and Salehi, 2007 ; Schroeder *et al.*, 2002). All isolates were resistant to Amikacin and that contrast results obtained by (Novotna *et al.*, 2005 ; Kim., 2005). Most isolates show intermediate susceptibility to Cefotaxime, Gentamicin, and Tetracycline. This may be due to mutations in the organism result from antibiotic selective pressure due to the lack of control on drug use in third world countries (Zhao *et al.*, 2001).

In the present study, all the *E. coli* O157:H7 isolates had the ability to produce haemolysis on sheep blood agar (carry enterohemolysin *hly* gene). This result agrees with results obtained by (Shalaby and Galab, 2008 ; Aslantas *et al.*, 2006 ; Kim *et al.*, 2005 ;

Novotna *et al*., 2005 ; Murinda *et al*., 2004) who found that 100%, 96%, 100%, 100%, 98% respectively, of tested *E. coli* O157:H7 isolates have haemolytic activity.

In the present study, the highest isolation rate of *E. coli* O157:H7 from diarrhea cases obtained in April (7.6%) followed by December (4.7%). while highest isolation rate from sheep was obtained in March (10%) followed by April (8.6%), February (5%) and November (4.7%). This result agrees with the results obtained by (Rahimi *et al*., 2008; Stanford *et al*., 2005 ; LeJeune *et al*., 2004 ; Murinda *et al*., 2002 ; Kudva *et al*., 1996) who found that highest isolation rate occurs in warm weather. In contrast, a study in Scotland (Ogden *et al*., 2004) referred that highest isolation rate was obtained in winter.

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