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RESEARCH ARTICLE

Using Polymerase Chain Reaction (PCR) Technique For Detection *E .coli O157:H7* BacteriaFrom Raw Cow's Milk and Local SOFT Chesses.

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Abstract

E.coliO157:H7 has emerged as an important gastrointestinal pathogen of man can cause severe illness and death. The present study was undertaken to detect the *E.coliO157:H7* in 125 samples (75 raw milk,25 pasteurized milk and 25 soft Arab chesses). Samples were collected from deferent location (source) in Baghdad city (Abu Ghraib, Al Kadhimiya, Al-A'amiriya and Fudaliyah). During the period from October 2015toJanuary 2016. All samples were screened to detect the presence of non-sorbitol fermenting colonies (NSF) on sorbitol MacConkey agar supplemented with Cefixime, Pastorium tolerate (CT-SMAC). A total of 125 samples, 28 (37.3%) from raw milk, 0 (0%) from pasteurized milk and 14 (56%) from soft Arab cheese (NSF), with chromogenic media 10(13.3%) from raw milk, and 4 (16%) from soft Arab chesses which appear purple on the media, *E. coli* isolates were serotyped as *E. coli O157:H7* by latex agglutination test, 7 (9.3%) isolates of raw milk, and 2 (8%) isolates of soft Arab chesses. All the latex agglutination positive isolates were positive to result of the vitek2 test. The specificity of the primers was evaluated byspecific primers (*uidR and flich7 genes)* 7(9.3%) 2(8 %) isolates of rawmilk, and soft Arab chesses samples respectively. In this study was evaluate based on Polymerase chain reaction (PCR) technique, that amplified the *uidR* and *flicH7* genes.

Introduction:-

Escherichia coli (*E. coli*), a bacterium that is a common inhabitant of warm blooded animals gut, including man. Most strains of *E. coli* are harmless; however, some strains such as *E. coli* O157:H7 can cause severe food bornedisease and are referred to as enterohemorrahgic*E .coli* (WHO, 1996).

E. coli O157:H7 is one of hundreds of gram negative *E. coli* and is the most well knownenterohemorrahgic strain (EHEC) (Peacock *et al.* 2001).

Enterohaemorrhagic*Escherichia coli* (EHEC) is the main cause of the recent outbreaks of diarrhea ,hemolytic uermic syndrome(HUS) and hemorrhagic colitis(Kwon and Cho, 2015).

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E.coli O157:H7 serotype are worldwide zoonotic and major foodborne pathogens responsible for the majority of severe cases of human enterohemorrhagic*Escherichia coli* (EHEC) diseases(Dulo,2014).

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).

Furthermore, E. coli O157:H7 has been recognized as a major food safety concern due to its lowinfectious dose. It is highly virulent, an inoculation of fewer than 10–100 CFU of E. coli O157:H7 is sufficient to cause infection (Coffey et al. 2011). E. coli O157:H7 pathogens were usually linked with a wide variety of foods such as milk, meats, meat products, dairy products and fresh products, contaminated from the field with wildlife droppings (Bell 2002; Fedio et al. 2011; Mora et al. 2007;Pennington 2010).

Raw milk was first recognized as a vehicle of transmission of *E. coli* O157:H7 in 1986(Martin et al., 1986; Oberst et al., 1998) when children from Wisconsin, USA, developed HC and HUS after drinking raw milk from dairy farms. Since then, there have been several reportsof outbreaks associated with this pathogen following consumption of raw milk (Borczyk et al., 1987; Doyle, 1991; Duncan et al., 1987; Karna et al., 2007; Mai et al., 1987).

The verocytotoxigenic*E. coli* (VTEC) enter the food chain via fecal contamination of milk, contamination of meat with intestinal contentsduring slaughter or contamination of fruit and vegetables by contact with contaminated manure. It is also transmitted through contact withinfected people, animals or animal waste. Moreover, contaminated water which used for irrigating or washing vegetables can also be source of infection for humans or animal (Paton and Paton, 1998).

Therefore, the present study was planned and aimed to detect and characterize *E. coli* O157:H7 from diarrheic and apparently healthy calves, raw milk and from children suffer from diarrhea, which can achieve throughisolation and identification of *E. coli* O157:H7. Serological detectionof *E. coli* O157by usingLatex agglutination and confirmation of the *E. coli* O157:H7 using polymerase chain reaction (PCR).

Materials and Methods:-

A total of one hundred fifty random raw milk and soft Arab chesses samples were collected from deferent location (source) in Baghdad city (Abu Ghraib, Al Kadhimiya, Al-A'amiriya and Fudaliyah). During the period from October 2015toJanuary 2016. The samples were brought to the laboratory oncrushed ice.

Culture conditions:-

Immediately in the laboratory, 25 ml of each sample was aseptically transfered to225 ml of modified tryticase soy broth (mTSB, Himedia, India) containing20 mg/ L novobiocin, followed by incubation at 18–24 h at 41.5 _C accordingto ISO 16654 (2001). The enriched culture were plated onto sorbitol MacConkey agar supplemented with cefixime (0.05 mg/ L) and potassium tellurite (2.5 mg/ L) (CT-SMAC, Oxoid,UK) and incubated at 41°C for 24 h. Non-sorbitol fermenting (NSF) colonies from each CT-SMAC plate were selected and streaked onto plates containing HiCrome EC 0157:H7 Selective agar Base and were incubated at 37°C for 24 h. Subsequently, one isolate from the subculture was further tested for agglutination with an E. coli O157:H7 latex test kit (Oxoid,UK) to detect the somatic antigen O157.

Molecular Detection of (uidRand flicH7) gene by using Multiplex PCRtechnique

was done by using commercially available DNA extraction and purification kit(Geneaid, Korea). The purified DNA was detected by electrophoresis in 1% agarose gelwith addition of ethedium bromide. Bromophenol blue stain added to the DNA sample and visualizes the DNA by U.V. light.

uidR: F:5-TGTTACGTCCTGTAGAAAGCCC -3
R: R:5-AAAACTGCCTGGCACAGCAAT -3
152bp (Bej et.al.,1991)
FlicH7: F :5-GCGCTGTCGAGTTCTATCGAG -3

R: 5-CAACGGTGACTTTATCGCCAT -3
625bp (Gannon et al.,1997)

This was done by using customize primers shown above. The PCR reaction mixture contains 12.5 µl of green master mix, 5 µl of purified bacterial DNA, 1 µl of each forward and reverse primers and 5.5 µl deionized water for total volume reaction (25 µl).

PCR tubes were transferred to the thermalcycler (after centrifuged for 10 seconds) to start theamplification reaction according to specific program for each gene. The results of the PCRwere 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 (Sambrook*et. al.*, 1989).

Two concentrations of agarose gel were prepared (1% and 1.5%) as we needed. The concentration of 1% agarose was used in electrophoresis after DNA purification process, while 1.5% 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 1.5% agarose gel containing 0.5 µl /ml 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.

Resultsand Discussion:-

According to the results of culturing on CT- SMAC, In the current study percentage of NSF *E. coli* was 37.3% of raw milk samples and 56% of soft Arab chesses shown in table (1). in the current study percentage of NSF isolates in raw milk samples was 37.3% which similar to the study contact by Roopnarine et al (2007) who showed that the percentage was 37.3%,but less than the percentage by Abbas et al (2012) which was 57.34% from 150 raw milk samples ,whereas higher than Abd Al-Azeem et al(2013)

who showed isolation percentage 6% . The explanation of difference in isolation percentage 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 current 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 broad range of contaminants including Proteus spp. Also it is necessary for the increase of bacteria to the level in which easily detected as low (10 cells) infection dose (Twardon et al., 2006). March and Ratnam, (1989) and Silveria etal. (1999) stated that the latex test is simple, highly efficient and reliable testin detecting E. coli O157:H7with 100% sensitivity and specificity. It was evident from the results shown in Table (1)

In the present study, the overall prevalence of E. coli O157:H7 latex agglutination test was 9.3% from raw milk samples This is convenient with the results showed by Al-Hasnawi (2010) Which was 11%. And less than that by Abbas et al (2012 ; Murinda et al., 2002 and Daood et al., 2007) .

which were 27.08%, 26.6% and 30.90% respectively, while it is much higher than results acquired by (Karns et al., 2007 and Belickova et al ., 2008) which were 0.23% and 1.02% respectively. In All theseprevious studies, the prevalence percentage were less than the results in the current study, this specific the presence of E.coli 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 positive percentage of E.coli O157:H7 in the present studyto latex agglutination test from soft Arab chesses (12%) was lessthan that byAbbas et al(2013) which was 15(33.34%) . The results showed that geographical or locational difference of thefarmer, contaminatedenvironmental conditions and unhygienicmeasures had an effect on the isolation percentage of *E.coli O157:H7* (Spano et al., 2003).

Table4-1:-Frequency of NSF and latex agglutination confirmed *E. coli* in raw milk and soft Arab chesses.

Isolates source	Total samples	Non sorbitol fermenting	O157 positive
Raw milk	75	28 (37.3%)	7 (9.3%)
Soft Arab Chesses	25	14 (56%)	2 (8%)
Total	100	42	9

In the current study *E.coli 0157:H7* was isolated from raw milkwith percentage 9.3% (7/75) andthat was higher than results obtained byAbbas et al(2012) the percentage of PCR positive for *vt1* of *E.coli*O157:H7 isolates from raw milk samples (4.67%) in Basrah city , Brenjchi 2011(0.77%) in Mashhad , Abdel-Fattah et al2008 (2.5%) inEgypt, and Abd Al- Azeem et al 2013(1%).

In this study *E.coli O157:H7* was isolation from soft Arab chesseswith percentage (8%) whichsimilar to the study contact by Panet*oet al* (2007) who showed that percentagewas (6%) in Turkey ,whereas higherthan the percentage by Abbas et al (2013) which was The percentage ofPCR positive for *Vt1* of *E.coli O157:H7*isolates of soft cheese was (3.34%) in Bashar city, but less thanthe percentage byVernozy- Rozand*et al* (2005) who showed thatpercentage was (13%) in France.

The high percentage recorded in the present study can be attributed to theuse the primers exploredin the assay were targeted againstthe *uidR*gene specific for all types of*E. coli* and the *fliCH7* gene specific for the h7 flagellar antigen of *E. coli* O157:H7. The multiplex PCR assaydeveloped was found to behighly specific as it produced PCR productsof 152 bp*E. coli* specific and 625 bp*E. coli* O157:H7specific (Kumaret al., 2013). The results showed that geographical or locationaldifference of the farmer, contaminated environmentalconditionsand unhygienic measures had an impactt on the isolationpercentage of *E.coliO157:H7* (Spano et al., 2003).

The rate of positive PCR isolates for *E. coli*O157:H7 in table 1:-

Isolates source	Total	*fliC*$_{H7}$ gene	*uidR*gene
Raw milk	75	7(9.3)	7 (9.3)
Soft Arab chesses	25	2(8%)	2(8%)
	100	9	9

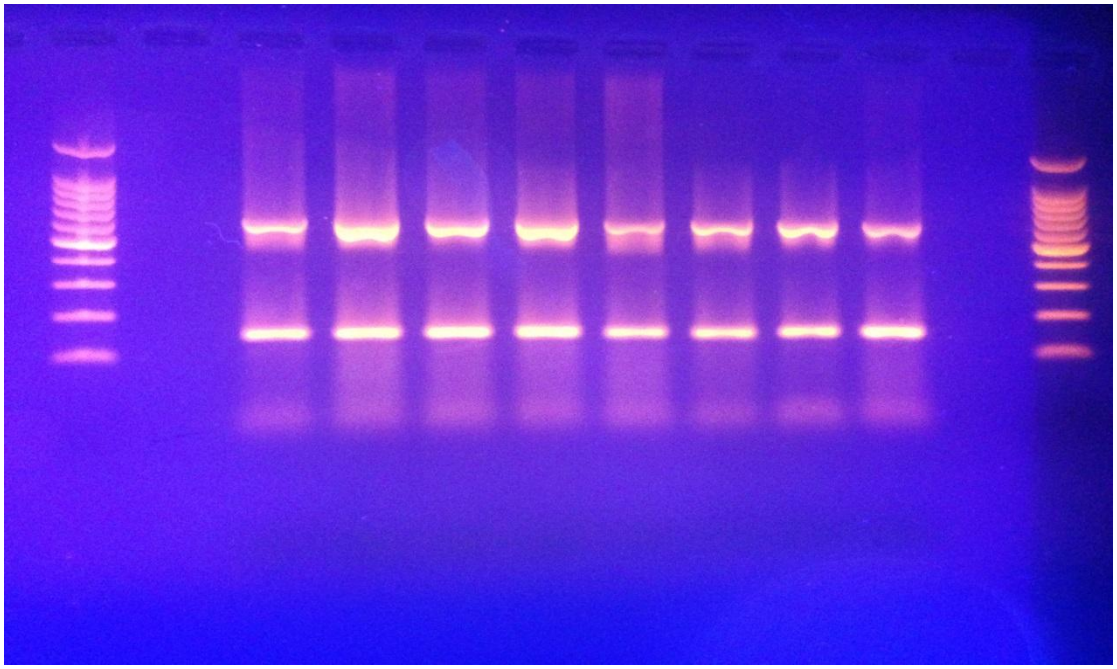

photo 1:- Gel electrophoresis image of amplicons by multiplex PCR fromselected *E. coli* O157:H7 strains.Showing the optimization of annealing temperature for *E.coli O157:H7 structural and virulence genes detection*using 1.5%

agarose gel at 8volt /cm for 2 hour. Lane 1,12: DNA ladder (100 bp),lane 2,11: negative control, lanes 3-10: amplicons obtained using 62 °C.

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