Rapid detection of Yersinia *enterocolitica* by using Real-Time PCR technique in some types of foods in Al-Qadisiya province

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(Received 13 April 2014, Accepted 22 May 2014)

Abstract

The present study was carried out for direct and rapid detection of pathogenic *Y. enterocolitica* in some types of foods such as (raw milk, local sweet cheese and minced beef meat) exist in local different markets of Al-Qadisiya province. A total of 192 samples were collected randomly from markets of Al-Qadisiya province involved 79 raw milk samples, 58 local sweet cheese samples and 55 minced beef meat samples. Real-Time PCR technique based SYBER Green dye were used for detection of this bacteria by amplifying invasion locus protein (*ail*) gene which is found especially in *Y. enterocolitica*, The results revealed that positive samples for this (*ail*) gene were 49 samples out of 79 examined raw milk (62%), also 6 samples out of 58 samples observed in local sweet cheese (10.3%) and 49 samples out of 55 samples of minced beef meat (89%). A total of positive samples were 104 out of 192 examined samples, samples of minced beef meat recorded highly significant contamination ($p<0.05$) as compared samples of raw milk which occupied second rank in contamination with pathogen and then samples of local sweet cheese which recorded less degree of contamination. The Real-time PCR technique was very specific and efficient for detection *Y. enterocolitica* in variety foods, when compared with culture method which have many problems.

Keywords: *Y. enterocolitica*, Real-Time PCR, *ail* gene, raw milk, sweet cheese, minced beef meat.

الكشف السريع عن اليرسينيا القولونية المعوية باستخدام تقنية تفاعل البوليمر السلسلي المؤلف في بعض انواع الغذاء في محافظة القادسية

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الخلاصة

اجريت الدراسة الحالية للكشف المباشر والسريع عن الـ يرسينا القولونية المعوية في بعض انواع الاغذية الموجودة في الاسواق المحلية في محافظة القادسية كالحليب الخام، الجبن الحلو المحلي، ولحم العجل المفروم جمعت 192 عينة عشوائياً من اسواق محافظة القادسية شملت 79 عينة حليب خام و58 عينة جبن حلو محلي و55 عينة لحم عجل مفروم. أستخدمت تقنية تفاعل البوليمر السلسلي المولف والمعتمد على صبغة السايبر غرين لكشف هذه البكتريا وذلك بمضاعفة بروتين الغزو الموضعي للـ (*ail*) جين والذي يتواجد حصرياً في بكتريا اليرسينيا القولونية المعوية. بينت النتائج ان العينات الموجبة للـ(*ail*) جين كانت 49 عينة من 79 عينة من الحليب الخام المفحوصة (62 %) و 6 عينات من 58 عينة من عينات الجبن الحلو المحلي المفحوصة (10.3 %) وفي 49 عينة من 55 عينة من عينات لحم العجل المفروم المفحوصة (89%) ، مجموع العينات الموجبة كانً 104 من 192 عينة مفحوصة. سجلت عينات لحم العجل المفروم أرتفاعا معنويا ($p<0.05$) في التلوث مقارنة بعينات الحليب الخام والتي جاءت بالمرتبة الثانية تلوثاً بالبكتيريا ومن ثم عينات الجبن الحلو المحلي والتي كانت اقل تلوثاً. وأظهرت النتائج ان تقانة تفاعل البوليمر السلسلي المؤلف تعد طريقة دقيقة جداً وفعالة في تشخيص الـ يرسينا القولونية المعوية في الاغذية المتنوعة مقارنة مع طريقة الزرع الجرثومي الاعتيادية والتي لها معوقاتها العديدة.

الكلمات المفتاحية: اليرسينيا القولونية المعوية ، تقنية تفاعل البوليمر السلسلي ، جين الـ ail ، الحليب الخام ، الجبن الحلو ، لحم العجل المفروم.

Introduction

Y. enterocolitica is a species of gram-negative coccobacillus–shaped bacterium classified belonging to Enterobacteriaceae, a pathogenic strain is characterized by a specific in the epidemiological mechanism of infections related with consumption of contaminated foods and associated with food borne disease called Yersinosis which characterized by diarrhea, mesenteric lymphadenitis, terminal ileitis, arthritis and septicemia (1, 2, 3, 4). The foods from animal origin play an important role in the transmission of *Yersinia spp*. (5, 6, 7). Pathogenic strain of Yersinia classified as psychrophilic bacteria because its ability to growing and multiplication in low-temperature of storage which used for keep of perishable food products. These reveal that the used of chilled and frozen storage of foods are potential reservoirs and sources of infection and may cause transmission of pathogenic Yersinia *spp.* (8, 9). The pathogenic potential of *Y. enterocolitica* is determined by complex interaction of chromosomal and plasmid genes (10). The presence of a 70 kb plasmid (pYV, plasmid Yersinia virulence) and specific chromosomal determinants (genes *ail, yst*, etc.) are necessary for the full expression of pathogenic potential (10, 11). Real-Time PCR is the developed method up to now based on *in vitro* amplification and has a potential for rapid and selective detection of microorganisms during the last years there is a significant progress in development of Real-Time PCR aiming at the quantitation of bacterial load in different complex samples, its principle based on detection of a fluorescent signal which is proportional to the number of amplicons in the tested sample, the main goal of the recent study is the optimization of a Real-Time PCR protocol based on the *ail* gene in order to quantify *Y. enterocolitica* (3, 12). This will contribute to the fast and quantitative detection of *Y. enterocolitica* in complex samples like meat, milk products and feces (1, 13). Detection of *Y. enterocolitica* in daily consummated food are important for consumer's health because inadequate healthy processing during preparation of these food materials (14). The aim of the present study was rapid detection of *Y. enterocolitica* by using Real- time PCR technique in some types of foods in Al-Qadisiya province.

Materials and methods

Samples collection

A total of 192 Samples of raw milk, local sweet cheese, and minced beef meat were collected randomly from different local markets in Al-Qadisiya province. The samples were collected in 25ml sterile containers and transported to laboratory and stored in a refrigerator until use for genomic DNA extraction accept raw milk which examined three hours after collection .

Genomic DNA extraction

Bacterial genomic DNA was extracted from milk, cheese, minced beef meat according method described by (15) by using (Genomic DNA extraction kit. Bioneer. Korea) with some modification. 1ml aliquot of milk was centrifuged at 6,000 × *g* for 10 min, and then the clear portion was pipette and discarded. The remaining milk solids and butter fat were used for DNA extraction, while, the cheese 250mg directly used for extraction. Whereas, 200mg from minced meat was homogenized by tissue lysis buffer that used for DNA extraction. The others steps were done according to kit instruction using DNA purification spin column. After that, the purified DNA eluted in elution buffer provided with kit and the extracted DNA was checked by Nano-drop spectrophotometer, then the DNA store at -20 °C until used for prepared of qPCR master mix.

Real-Time PCR

Real-Time PCR technique was used to detection of *Y. enterocolitica* food borne bacteria using SYBER green dye based amplification of attachment invasion locus protein (*ail*) gene in *Y. enterocolitica* from milk, cheese, and minced meat. This technique was carried out according to method described by (3, 12). Real-Time PCR primers were designed by this study from conserved region attachment invasion locus

protein (*ail*) gene (GenBank: JX972144.1) using NCBI-GenBank data base and primer3 plus online, and these primers were provided by Bioneer Company. Korea as showed in table (1).

Table (1): structure of primer

Primer	Sequence		Amplicon
ail	F	GGATGCAATTCAAC CCACTTCC	98bp
	R	TGCACCAAGTATCCA AGTGC	

The Real-Time PCR amplification reaction was done by using (AccuPower™ 2X Green star qPCR master mix kit, Bioneer. Korea) and the qPCR master mix were prepared for each sample according to company instruction as in table (2).

Table(2) Composition of qPCR master mix

qPCR master mix	Volume
DNA template	3µL
2X Green star master mix	25µL
ail Forward primer (10pmol)	1.5µL
ail Reverse primer (10pmol)	1.5µL
DEPC water	19µL
Total volume	50µL

These qPCR master mix reaction components that mentioned in table were placed in sterile white qPCR strip tubes and transferred into Exispin vortex centrifuge for 3minutes, the place in Miniopticon Real-Time PCR system and applied the following thermo cycler conditions as in the table (3).

Table (3): Thermo cycler conditions

qPCR step	Temperature	Time	Repeat cycle
Initial Denaturation	95 °C	3 minutes	1
Denaturation	95 °C	10 sec	40
Annealing\ Extension	58 °C	30 sec	
Detection(scan)			
Melting	60-95°C	0.5 sec	1

Statistical analysis

The data were analyzed using Chi-square ($p<0.05$) to detect significant differences among each groups (16).

Results

The results of Real-Time PCR assay for detection of contamination with *Y. enterocolitica* shown from 192 examined samples 104 were positive with percentage (54.1%), this results distributed to 49 positive raw milk samples from 79 examined samples with percentage (62%), while 6 samples were positive from 58 examined samples of sweet local cheese with percentage (10.3%), and 49 samples were positive from 55 examined minced beef meat with percentage (89%) (Table 4).

Table (4): The occurrence of pathogenic *Y. enterocolitica* in some types of foods.

Items	No. of tested samples	No. of positive samples	%
raw milk	79	49	62 [b]
Sweet local cheese	58	6	10.3 [c]
Minced beef meat	55	49	89 [a]
Total	192	104	54.1

[abc] The different superscript letters in the column refer to the significant differences ($p<0.05$), χ^2 cal. = 59.633.

The contaminated samples of minced beef meat recorded highly significant differences ($p<0.05$) as compared to other examined samples.

The direct detection of *Y. enterocolitica* by Real-Time PCR based SYBR Green dye was appeared highly sensitive and specific for amplification of attachment invasion locus protein (*ail*) gene and the specifity was determined by dissociation curve (Melt Curve). Where the positive amplification product samples were showed specific amplification at melt peak mainly at (Tm: 80C°) without primer diamer or nonspecific products (Fig.1,2).

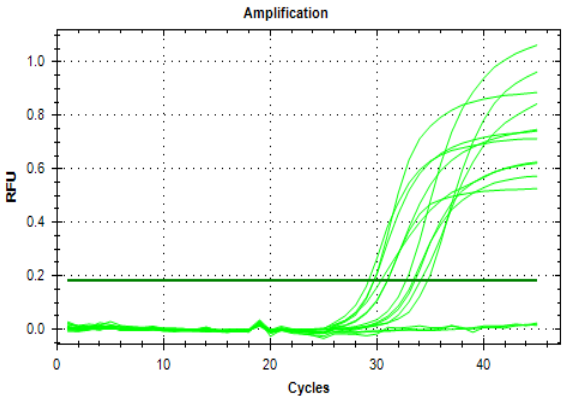

Fig. (1): Amplification plot of *Y. enterocolitica* attachment invasion locus protein (*ail*) gene by Real-Time PCR in some type of foods.

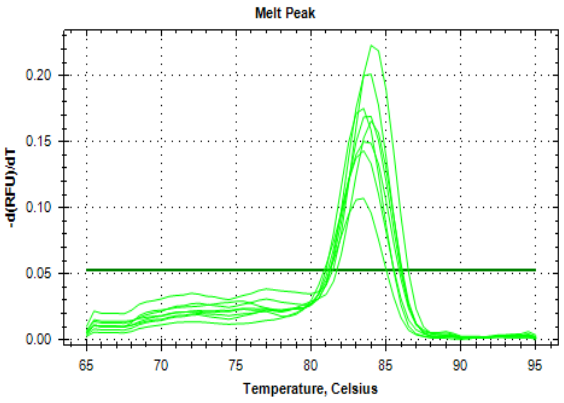

Fig. (2): Melt curve of *Y. enterocolitica* attachment invasion locus protein (*ail*) gene by Real-Time PCR in some type of foods. Shows the specifity of gene amplification at (Tm: 82.5°C).

Discussion

Real–time PCR method is a specific and rapid technique for rapid detection of *Y enterocolitica* in different foods (1, 17). The designed primers of attachment invasion locus protein *ail* gene in this study were chosen as an excellent target for pathogenic *Y. enterocolitica* because their limited virulence strains of *Y. enterocolitica* rather than nonpathogenic *Yersinia spp.* these results are in accordance with (10) who attributed that to heterogenisity within the bacterial population for the presence of the virulence plasmid and associated with human pathogenicity. (11,18) refer to the presence of pathogen which related with *ail* gene was recommended in Europe and America and other world countries which associated with a pathogenicity of human. Conventional PCR does not differentiate between *Y. enterocolitica* and *Y. Pseudotuberculosis* but only Real time PCR method is developed for detection to *ail* gene which is found in pathogenic *Y. enterocolitica* as pointed by (2,13). Also (6) refers to the contamination of variety of foods occurs at high levels and this need a highly specific and rapid method for detection of indictor pathogen. In addition to that, (18) reaches to, there are many problems during culturing of this *Y. enterocolitica* because doesn't not well compete with other pathogenic species. High numbers of other species of Enterobactericae inhibit growth of *Y. enterocolitica* has found by (10). Highly presence of bacteria in raw milk resulted from bad handling during milking and storage of milk in contaminated utensils where the bacteria transfer from the farm to the market and without use heat treating, similar results were obtained by (1). In local sweet cheese recording low level of contamination, this result can be attributed to heat treating of raw milk during processing of making cheese, contamination happens during handling but that will not decrease the risk of this food because of eating directly; this accordance with (3). Highly contamination of minced beef meat samples resulted from high contamination in slaughterhouse and contaminated water which was used for washing of carcasses in addition that bad handling with meat in the local markets. This results similar to that reported by (7,12). In public health field when the indicator bacteria as *Y. enterocolitica* was detected in any type of foods this means that food unfit for human consumption.

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