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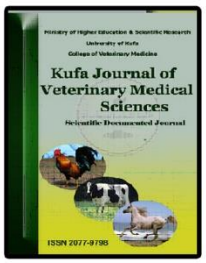

PCR for direct detection food pathogen *Listeria monocytogenes* and Enteropathogenic *Escherichia coli* from milk and milk products in Al-Diwanyia city of Iraq

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Abstract

The current study was carried out to directly detect *Listeria monocytogenes* as well as enteropathogenic *Escherichia coli* bacterium from daily consumed milk and milk products (buffalo raw milk, local cheese, and local cream) which were collected randomly from different markets of Al-Qadissiya province. In this study the PCR techniques were employed on the basis of amplifying virulence factors (*bfpA* and *hly* genes) in *L. monocytogenes* and enteropathogenic *E. coli* respectively. Analysis of the results showed the highest prevalence of *L. monocytogenes* (14.2%) in local cheese which was followed by 6.2% in buffalo raw milk and less prevalence (3.4%) were found in local cream. Analysis of the results of enteropathogenic *E. coli* revealed highest prevalence (37.5%) in buffalo raw milk while local cheese and local cream showed the same prevalence (20%). This study successfully developed and used PCR technique that was prompt and sensitive/specific in detecting food born pathogenic *L. monocytogenes* and enteropathogenic *E. coli.* This technique could be used as an alternative to the other methods, making it possible to identify the risk for these pathogens on public health.

Key word: PCR, Listeria monocytogenes, Escherichia coli, milk, milk products.

الخلاصة:

اجريت الدراسة الحالية من اجل الكشف عن بكتريا اللستريا *Lmonocytogenes* وبكتريا الامعاء القولونية *Escherichia coli* التي تتواجد يوميا في الحليب ومنتجاته التي تشمل (حليب الجاموس الخام ،الجبن المحلي والكريم المحلي) والتي تم جمعها عشوائيا من اسواق مختلفة في محافظة القادسية، في هذه الدراسة تم استخدام تقنية سلسلة البلمرة على اساس التضخيم منن عامل ضراوة الجين (*bfpA*) والجين (*hly*) في اللستريا و البكتريا القولونية .

اظهرت النتائج ارتفاع في ضراوة بكتريا اللستريا (14.2%) في الجبن المحلي وبعده (6.2%) في حليب الجاموس الخام وظهرت اقل ضراوة في الكريم المحلي (3.4%)، في حين ظهرت نتائج ضراوة بكتريا الامعاء القولونية ارتفاع في حليب الجاموس الخام (37.5%) ونفس النتيجة في الجبن المحلي والكريم المحلي (20%) كان الغرض من هذه الدراسة وضع الـ PCR كطريقة سريعة ومحددة للكشف عن الممرضات الغذائية (اللستريا والبكتريا القولونية)والتي يتم استخدامها كبديل للطرق االاخرى ويجعل من الممكن التعرف على خطر الممرضات على الصحة العامة .

Introduction

Recently, an increasing interest have been reported amongst certain consumers towards a decrease in processing of foods, not excluding raw milk and milk products (1,2), on the bases of demand from consumers that pasteurization process could destroy non-heat stable nutrient as well as vitamins that are useful to humans. Extensive investigations (3,4) have found that *E. coli* O157:H7, *Listeria monocytogenes* as well as Salmonella spp. have been isolated in 2 to 6% of bulk raw milk samples. Even though these pathogenic microbes are destroyed if the milk is pasteurized, their virulence is retained in unpasteurized milk. In the United States, more than 93 epidemics emerging from foodborne pathogens as a result of consuming unpasteurized milk or milk products have been reported in 2009, leading to 1,837 sicknesses, 195 cases of hospitalization, and 2 mortality (5). Higher frequencies of epidemics as well as mortalities associated with unpasteurized milk products have equally been documented in Europe (6). The tradition techniques of bacterial isolation has restricted capability of scholars in studying the safety of foods in associations with the consumption of unpasteurized milk, not excluding the pathogen contamination levels over a period of time. The traditional culturing techniques are done on the bases of enriching one or several liquid media which are subsequently plated on selective solid media. Rapid result may be acquired utilising selective immunology techniques or PCR techniques (7). Immunoassays are available for direct quantitation of pathogens in milk and foods, but their detection is limits are orders of magnitude higher than cultural methods. PCR technique is capable for detecting single organisms rapidly and quantitatively, but several requirements must be met to achieve this in practice (8). Although many of PCR methods for detection of pathogens in milk are available, existing methods lack one or more of these features. Features of a number of direct PCR methods with detection limits of 10 cfu /mL and many work large sample volumes and most utilize a large fraction of the extracted DNA, providing high potential sensitivity. The aim of percent study to use highly specific and rapid PCR technique for direct detection important food borne pathogen from milk and milk products in Al-Diwanyia city of Iraq.

Materials and Methods

Samples collection:

32 raw buffalo milk, 35 local cheese, and 30 local cream samples were collected from different markets in Al-Diwanyia city. The samples were collected in 25ml sterile and transported into laboratory and stored in a refrigerator until use for genomic DNA extraction

DNA extraction and PCR Method:

PCR technique was performed for food borne pathogen (Listeria monocytogenes and Enteropathogenic Escherichia coli) by using specific primers for virulence factor (bfpA gene) and (hly gene) respectively. As following steps:-

1-DNA extraction: The milk samples were subjected to bacterial nucleic acid extraction by using commercial DNA extraction kit (Presto Mini-DNA Bacteria Kit. Geneaid Biotech Ltd. USA) and according to method described by Quigley (9)**.** The extraction method was don depend on the manufacturing instructions by using gram positive bacteria DNA Protocol extraction method by using (20 mg/ml) lysozyme buffer.

2-Nanodrop: The extracted DNA was estimated by nanodrop device at 260/280nm, and then kept at deep freezer until used in PCR method.

3-Primers: The PCR primers were designed in this study using NCBI Gene sequence data base (Bfp gene: GenBank: AF119170.1 hly gene: GenBank: M24199.1) and primer 3 plus design. These primers were provided from Bioneer company, Korea as following table (1):-

Table (1):- PCR primers mecA gene:

Primer	Sequence (5'-3')		Amplicon
Bfp gene	F	TGCGGATGGATGCCTTTTCT	266bp
	R	GAGCTGAGTCGTTCACTGCT	
hly gene	F	CACCAGGAGTTCCCATTGCT	405bp
	R	TGGTGCCCCAGATGGAGATA	

4- PCR master mix preparation: the mix was prepared using (Accu-Power®PCR-PreMix-Kit) master mix reagent and done depend on company instructions as following table (2)

Table (2) company instructions of PCR master mix:-

Master mix	Volume
DNA template 10ng	5µL
Forward primer (10pmol)	1.5µL
Reverse Primer (10pmol)	1.5µL
PCR water	12µL
Total volume	20µL

After that, the PCR mix that revealed in table above placed in AccuPower PCR -PreMix that contain all other PCR components which needed to reaction such as (Taq DNA polymerase, dNTPs, 10 PCR buffer). Then, all the PCR tubes transferred into vortex centrifuge for 3 minutes. Then transferred into thermocycler (MyGene, Bioneer. Korea).

5- PCR thermocycler conditions:

Table (3) PCR thermocycler conditions

PCR step	Temp.	Time.	Repeat cycle
Initial denaturation	95˚C	5min.	1
Denaturation	95˚C	30sec.	30 cycle
Annealing	58˚C	30sec.	
Extension	72˚C	30sec.	
Final extension	72˚C	5min.	1
Hold	4˚C	Forever	-

6- PCR product analysis: The PCR products were examined by electrophoresis in a 1% agarose gel using 1X TBE buffer, stained with ethidium bromide, and investigation under UV transilluminator.

Results and Discussion

The Polymerase chain reaction PCR was appeared as sensitive and specific assay in direct detection of Listeria monocytogenes and Enteropathogenic Escherichia coli from milk and milk products as following table:

Examined food	No. of samples	Positive samples L. monocytogenes	Positive samples E. coli
local cheese	**35**	**(5/%14.2%)**	**(7/20%)**
Buffaloes raw milk	**32**	**(2/6.2%)**	**(12/37.5%)**
Local cream	**30**	**(1/3.3%)**	**(6/20%)**

The PCR amplification of BfpA gene and hly genes in positive samples was shown clear PCR product bands on Agarose gel electrophoresis at 266bp BfpA and 405bp hly PCR products. Figure (1) and Figure (2).

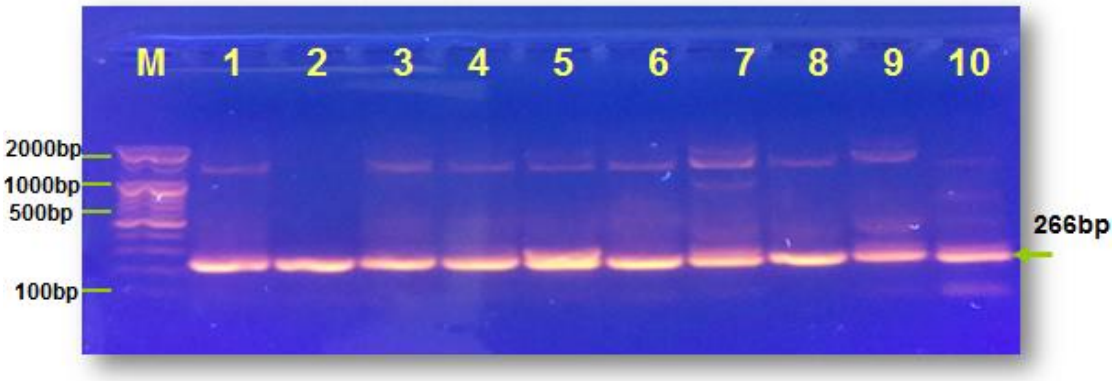

Figure (1): Agarose gel electrophoresis of PCR assay show the positive results of BfpA gene in Escherichia coli. Where, Lane (M) DNA marker (100bp), Lane (1-10) positive samples for BfpA gene at PCR product size.

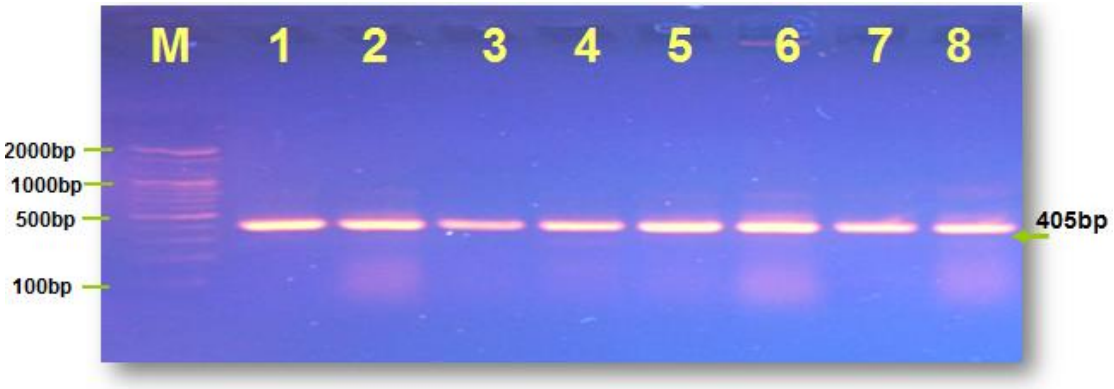

Figure (2): Agarose gel electrophoresis of PCR assay show the positive results of hly gene in Listeria monocytogenes. Where, Lane (M) DNA marker (100bp), Lane (1-8) only positive samples for hly gene at 405bp PCR product size.

The main objective of present study was to evaluate the possible risk that might occur as a result of consuming of raw buffalo milk or milk products by direct detection of two main food borne pathogenic bacteria, PCR technique results revealed that an overall high prevalence of *Escherichia coli* (25at 25.77%). Pervious study at first time in Iran was evaluated of prevalence *Escherichia coli* (EPEC) from raw animal milk. Who recorded the decrease contamination of milk with EPEC of 206 samples, 17 (8.25%) (10).

While the present result of overall *Listeria monocytogenes* (8/ at 8.24 %) in raw buffalo milk or milk products when compared to the other study quite higher prevalence of Listeria *monocytogenes* in milk was observed at 21.32% (11), 22% (12), 26.13% (13), and 54.7% (14). The increased level of detection by using PCR technique when compared with conventional methods may be characterized to the high sensitivity of PCR in addition to inhibitory effects of raw milk components on recovery of bacteria during isolation which is due to presence of high level of lactoferrin (15). Conventional culture based methods are labour intensive and time consuming, in many instances requiring more than days to complete (16). Molecular identification of pathogenic *Escherichia coli* and *Listeria monocytogenes* it was based use of virulence genes such as (structural *bfpA* gene that encoded the bundle-forming pilus of EPEC) and (*hly* gene that encoded the hemolysin of L.monocytogenes) respectively by using PCR due to many studies who suggesting the pathogenic potential of these isolates (17, 18). In conclusion the use of PCR is rapid and sensitive molecular tool for detection of pathogenic *Escherichia coli* and *Listeria monocytogenes* in raw buffalo milk and milk products that has proven to be of great diagnostic value.

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