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Detection Of Some Foodborne Pathogens In Some Food Samples Using Species - Specific Polymerase Chain Reaction Technique

Sakar kamal Hamasaleh & Khulod Ibraheem Hassan

Food Sciences department, College of Agricultural sciences, Sulaimani University
E. Mail. khlood.hassan@univsul.edu.iq

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Abstract

Polymerase Chain Reaction (PCR) techniques was applied as a rapid method for detection of some food-borne pathogens directly without culturing, to detect three species of bacteria, including: *Shigella flexneri, Escherichia coli 0157:H7* and *Listeria monocytogenese* by targeting specific genes for each pathogen from cultures of various types of artificially inoculated foods, which were spiked with reference bacteria at known concentrations. DNA was isolated from each food sample using phenol -chloroform based method.Positive results were obtained which produced specific amplicons of the expected sizes of each of them (691 bp in *L. monocytogenese*, 556 bp in *E.coli o157:H7* and 600 bp in *S. flexneri*). The detection limit of the assay were 10^3 CFU/ ml For *L. monocytogenese*, and 10^4 CFU/ ml for both *S. flexneri* and *E.coli o157:H7*. overall results of this study indicate that PCR is a good tool for rapid screening of the three pathogens in food and this application can be followed for detection of any pathogenic bacteria transmitted through food.

Introduction

Bacterial contamination of food represents one of the main public health problems worldwide, because it caused many food borne diseases, which some of them were lethal. The detection of pathogens in food is an important component of any integrated program to ensure the safety of foods throughout the food supply chain. Traditionally the method for detection and identification of bacteria based on specific morpholological and biochemical tests after their isolation in differential and selective growth media ***[1]***, although these methods can be sensitive, inexpensive and give both qualitative and quantitative information on the number and the nature of the microorganisms tested, they are greatly restricted by assay time ***[2]***, especially with the food samples of short shelf life, due to all the steps needed for confirmation of presumptive positives results which are frequently not available until the food has been released to the market increasing the risk of pathogen transmission. Furthermore, pathogens are often present in food in very low numbers with a high background of indigenous micro flora, thus make the recovery of target organisms difficult ***[3]***. Hence, extensive research has been carried out to overcome these problems, which required developing diagnostic tests that can specifically detect the target pathogen rapidly by using nucleic acid–based assays for the detection and identification of food borne pathogen ***[4]***. Among them polymerase chain reaction (PCR) has distinct advantages over traditional methods for detecting and identifying bacteria in foods, in term of

specificity, sensitivity, rapidity, accuracy and capacity to detect small amounts of target nucleic acid in a sample, beside it based on DNA which is more stable than proteins to high temperatures, high pH, organic solvents, and other chemicals; hence samples can be treated in a relatively harsh manner without destroying the nucleic acid for detection ***[5]***. Thus , it is highly recommended in order to obtain more precise and reliable result ***[6]***.

Many PCR techniques have been validated to make PCR a standard tool used by food microbiology laboratories to detect pathogens in foods ***[1***; ***7]***, and numerous studies have been published on PCR detection of pathogenic bacteria in food including *Shigella spp.* ***[8***; ***9]*** which has been identified as one of the most important agents of diarrhea ***[10]*** and Shigellosis ***[11]***. Shigella survives in various foods, under optimal temperature conditions, and without severe acidity, they may be recovered from milk, eggs, cheese, beside other associated foods such as salads, raw vegetables, and poultry. PCR also used for detection of *E. coli* O157: H7 ***[12***;***13]***, as it is robust pathogenic microorganism that can cause several human food borne disease . It can adapt to and survive in a wide range of environmental conditions, including shifts in temperature, low pH and desiccation ***[14]***. Hence incidence rate involving it in food samples is on rise. Cattle have been identified as a principal reservoir of *E. coli O157:H7****[15]***. Studies have shown than up to 30% of all cattle are asymptomatic carriers of *E. coli O157:H7* ***[16***; ***17]***. Other studies applied PCR for detection of *Listeria monocytogenese* in food ***[18***; ***19]***. The public health hazard views *L. monocytogenes*e as one of the major food pathogens ***[20]***, *L. monocytogenese* cause listeriosis outbreaks in both Europe and North ***[21***; ***22]***. The frequent occurrence of *L. monocytogenese* in food especially in meat and chicken may pose a potential risk for consumers ***[23]***. The ability to formation of biofims causes *L. monocytogenese* to persist in the food processing line. Few studies deals with the application of PCR for detection of pathogenic bacteria in Kurdistan region of Iraq , Hence, this study was conducted with the aims of optimize methods based on the gene specific PCRs, for the detection some pathogenic bacteria from food samples, which artificially contaminated with the pathogenic bacteria to be tested through PCR methods and determine their sensitivity to develop effective prevention and control strategies for these pathogens.

Materials and methods

A. *Bacterial strains*

Strains of bacterial used in this study(Shigella flexneri 14028, E.coli o157:H7 and Listeria monocytogenese 25923,) were obtained from the Medya Diagnostic Centre (MDC) in Erbil, Kurdistan region-Iraq. Bacterial strains were cultured for 24 hrs in 10 ml of tryptic soy broth yeast extract broth (TSBYE) at 35°. The TSBYE contained 30 g of tryptic soy broth powder , 6 g of yeast extract, and 1 liter of water ***[24]***.To study of the sensitivity of PCR, serial decimal dilutions of the cultures in phosphate-buffered saline (PBS) were plated onto trypticase soy agar (TSA) for each bacterium. The plates were then incubated at 37 ºC for 48 hours before enumeration. One ml of each dilution was used to inoculate 25g of each food sample. Then each inoculated sample was placed in 225 ml of TSBYE medium and homogenized using a stomacher at 90s ***[25]***. The cultures were incubated for 6h at 36 ºC. After incubation, bacterial DNA was extracted from each culture.

B. DNA extraction

Ten ml from each samples including (chicken meat, beef meat and lettuce) were used for DNA extraction according to the method developed by Sambrook and Russel ***[26]*** with some modification as follows: the pellets of 24 hours broth media were re-suspended in 2 ml of TE buffer (500 mmol Tris-HCl [pH 8.0], 100 mmol NaCl, 1 mmol sodium citrate, 5 mmol EDTA) ,then five mg/ ml lysozyme added and incubated for 1 h at 37°C with occasional agitation, after that three cycles of freezing and thawing in a 65°C water bath were included to facilitate cell wall destruction, and

releasing of nucleic acids, then Proteinase K was added to a final concentration of 2 mg /ml, and the mixture incubated for an additional 30 min with occasional agitation, then thirty µl SDS(10%) were added and the solution mixed gently by inversion, incubated for 30 minutes at 37 ºC. Then an equal volume of phenol- chloroform iso amyl was added then centrifuged for 5 minutes. Nucleic acids were precipitated via addition of ammonium acetate and an equal volume of isopropanol alcohol, centrifuged at maximum speed in a micro centrifuge for 20 min. Pellets were washed with 70% ethanol and air-dried at 37ºC for 5 min, then visualized using gel electrophoresis **.** The purity of isolated DNA determined by estimating the concentration of the DNA and Proteins in each samples using spectrophotometer

C. PCR analysis

The primers sequences which were provided by Bionerr Company (Korea) were as follows: Fwd:GCCGGTCAGCCACCCTCTGAGACTAC, Rev:GTTCCTTGACCGC CTTTCCGTACCGTC for *Shigella flexneri* and the second pair of primers was Fwd: CGA GGG CTT GAT GTC TAT CAG , Rev: TCA GTA TAA CGG CCA CAG TCC that amplify 556 bp in *E.coli O157:H7* ***[27]***, and the third pair of primers was Fwd:AGGGCTTCAAGGACTTACCC, Rev:ACGATT TCTGCTTGCCATTC that amplify 691bp in *L. monocytogenes* ***[22]***.The reagents required for PCR reaction include 20µl reaction mixture (contained 1U of Taq DNApolymerase,10 mM of Tris-HCl (pH9.0),30 mM of KCl, 1.5 mM $MgCl_2$, each dNTPs (dATP, dCTP, dGTP, dTTP)) with template DNA (25-50ng)and primer (10 pmol) . Then the products were run on 1.2% agarose gel electrophoresis and stained by Ehidium bromide to visualize the amplified fragmen under UV . The program was setup as follow : One cycle for 94 ºC for 2 min and thirty cycles: Step 1: 95 ºC for 1 min ,Step 2 annealing temperature *was 55* ºC *using the E.coli O157:H7* and *Shigella flexneri* gene targeting primers and 54 ºC using L. *monocytogenes* gene targeting primers for 1 min ,Step 3: 72 ºC for 2 min and One cycle of 72 ºC for 5 min (Final extension) . In order to evaluate and verify the specificity of PCR protocol for each strain, the primer pairs was tested by PCR on DNA templates prepared from a panel of five different bacterial isolates including *Salmonella* typhimurium, *Shigella flexneri*, *Listeria monocytogenese*, *E.coli O 157:H7* and *Staphylococcus aureus.*

D. Detection of PCR sensitivity

To determine PCR sensitivity, all strains were grown for 24 h in 10 ml of universal culture medium (tryptone soya broth yeast extract) (TSBYE) at 37 ºC. Various concentrations (10 -10^4 CFU/ml) of each pathogen, was added as 1ml to 9 ml of homogenate food samples ***[24]***. Then the bacterial mixtures from each dilution were harvested by centrifugation at 4000 rpm for 20 min and analyzed by preparing DNA as described previously. Detection probability was calculated as positive PCR results corresponding to particular concentrations of bacterial suspensions determined as colony-forming unit per milliliter by the plate count method.

Result and discussion.

A. DNA Isolation

The results shown that total DNAs extracted using the protocol of Anonymous ***[28]*** provided a sufficient quantity of bacterial DNA suitable for PCR amplification. The purity of DNA isolated in this study ranged between 1.7-1.8 . The method applied for genomic DNA extraction here was effective in obtaining the genomic DNA of pathogenic bacteria ***[29]***. This method has been used previously to extract genomic DNA from *E. coli O157:H7* ***[30]***. The importance of this method is that few steps are required which make it rapid and minimize the possibility of contamination, and because this method is not specific for a particular organism, thus it may be useful for detecting other bacterial pathogens. Besides , the advantage of this method resided on several components in the extraction buffer that helped in isolation of a good yield and pure DNA, including: lysozymes , known as muramidase or N-acetylmuramide glycan hydrolase, these enzymes are glycoside hydrolyses, that

damage bacterial cell walls by catalyzing hydrolysis of 1,4- ß linkages between N-acetyl muramic acid and N-acetyl-D-glucosamine residues in a peptidoglycan and between N-acetyl-D-glucosamine residues in chitodextrins ***[31]***.

B. The specificity of PCR reaction

The analyses indicated that the primer pairs were specific for their corresponding target organisms, and the primers specifically amplified 691 bp target the *lmo0737* **gene** in *L. monocytogenese*, 556 bp target the ***Stx2A*** **gene** in *E.coli o157:H7* and 600 bp from *ipaH* gene in *Shigella. flexneri* , it mean that they did not give any false positive results, These results were parallel to those obtained by Oliveira *et al.* ***[32]*** and Nucera *et al.* ***[33]***. Information on the target sequence is an important parameter for obtaining successful result of PCR reaction ,PCR detection of virulence genes in Shigella such as *ipaH,* are considered a good and specific diagnostic tool of Shigella from clinical and food samples ***[34***; ***35***; ***36]***. The *ipaH* loci comprise a multicopy antigen gene family unique to Shigella species and enteroinvasive *Escherichia coli (*EIEC*).*The protein of this gene was required for efficient escape from endocytic vacuoles ***[8]*** and inhibit nuclear factor *kappa B* (*NFkappa B*) dependent gene expression that is responsible of the immune response of the host cell ***[37]***. The primers that used for detection of *L.monocytogenes* in this study target the *lmo0737* gene; which was previously used by Toyotome *et al*. ***[38]*** and Doumith *et al.* ***2]***, to specifically amplified *L. monocytogenese lmo0737* gene.

C. Detection of PCR sensitivity

To test sensitivity of the PCR assay for detection of *Shigella flexneri* templates were prepared by the decimal serial dilution of the bacterial culture, then extracting DNA from each dilute after enumeration of the cells, the products from DNA amplification by PCR are shown in Figure 1.The results showed that after inoculation with 10^2, 10^3, 10^4, 10^5 CFU/ ml of *Shigella flexneri* in food samples, bands of 600 bp (*ipaH*) were observed using 10^4 CFU/ ml , mean that the detection level of Shigella in lettuce by PCR was 10^4 CFU/ ml . Heijnen and Medem ***[39]***.and Theron *et al.* ***[40]*** also identified virulent *Shigella flexneri* and entero invasive *E. coli* (EIEC) in spiked environment water samples by using PCR method, with detection limit of 1.6×10^3 CFU /ml.

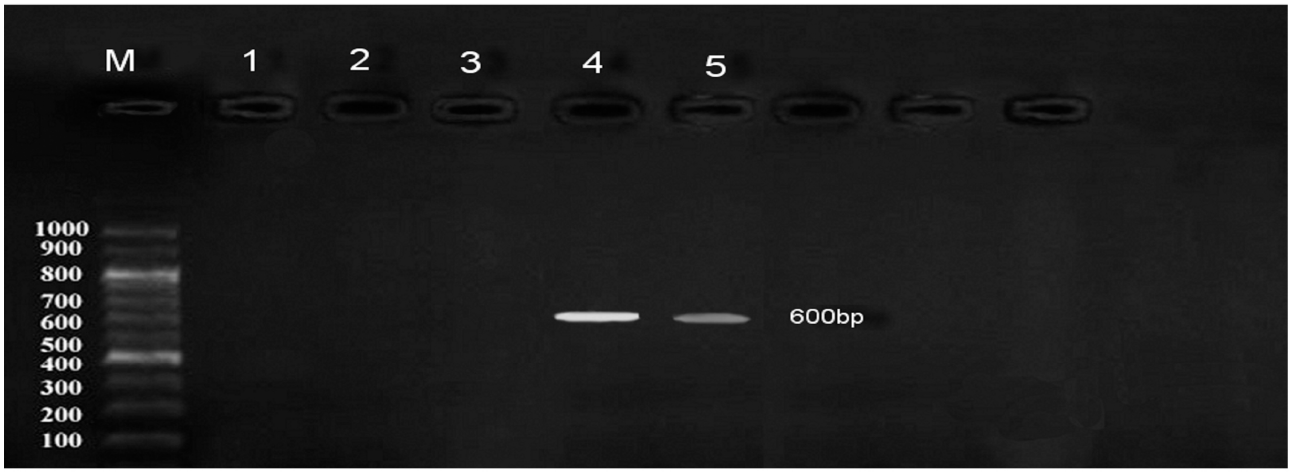

Figure 1: Sensitivity of the PCR for the detection of *Shigella flexneri*, the first Lane represents DNA ladder 1 kb; Lanes 1 to 4: target gene isolated from 10, 10^2, 10^3, 10^4 CFU/ ml, respectively.

To evaluate the ability of the PCR assay to detect *E. coli O157:H7* that had been inoculated into food samples, different concentrations of *E. coli O157:H7* cells were used which suspended in TSBYE broth. The detection limit in a concentration of 10^4 CFU/reaction gave positive results with ground

beef samples (Figure 2), faint bands were observed using the concentration of 10^3 and no band was observed in 10^2 CFU/ml concentrations, whereas Cui ***[41]*** record the detection limit for *E. coli O157:H7* cells suspended in pure saline was 1.34×10^5 CFU /ml , and this may attributable to substances in the sample inhibitory to PCR reaction. Hence the detection system that based on PCR methods can detect cells that are non-cultural in contrast to culture-based methods, which may result in false-negatives as *E. coli O157:H7* has been reported to enter a viable, non-cultural state under some environmental conditions ***[42]*** which may lead to incorrect results, however sometimes false-negative results were obtained using PCR methods because the test was detect a certain subtype of *E. coli O157:H7.* A wide range of genetic variability has been identified among *E. coli O157:H7* strains ***[43]***.Therefore, genetics-based tests may focus on a target that is present in all O157:H7 strains, false positives would also be expected in any PCR assay because dead cells or free DNA would also be detected and it does not necessarily indicate that a sample has live pathogenic bacteria, and this could be advantageous for some purposes. For example, PCR could be useful in tracing the source of an outbreak to identify Shiga toxin-producing E. coli (STEC) in a sample.

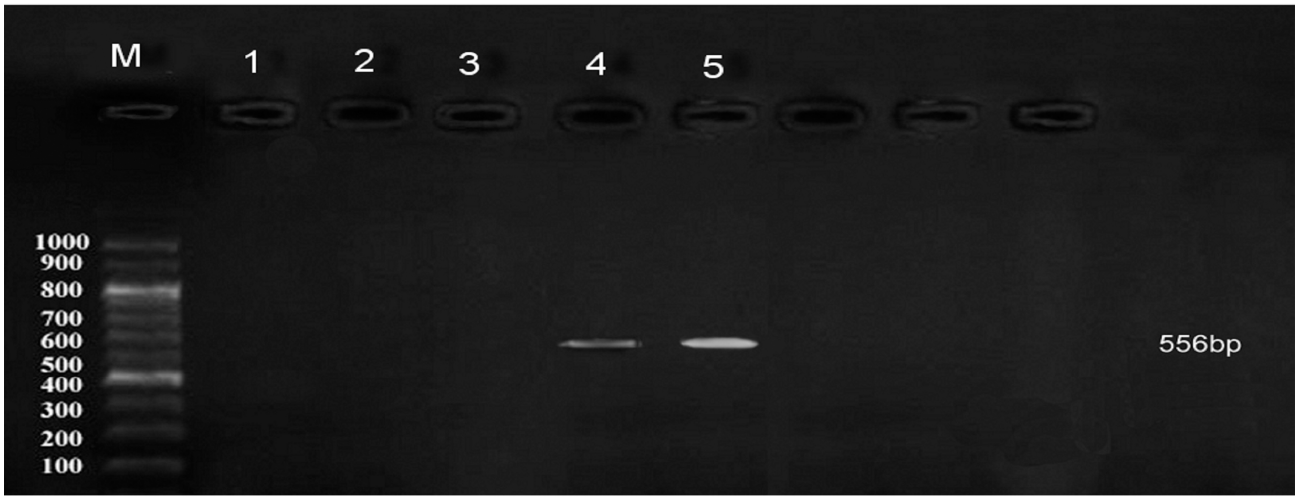

Figure 2: Sensitivity of the PCR for the detection of *E.coli O157:H7,* the first Lane represents DNA ladder 1 kb; Lanes 1 through 4: target gene isolated from 10, 10^2, 10^3, 10^4 CFU/ ml, respectively.

In estimating the sensitivity of PCR for detection of *Listeria monocytogenese* , The results are shown in Figure 3 which represent the gel electrophoresis of the PCR products that amplified desired PCR products of 691 bp band specific for *L. monocytogenese* gene (lmo0737) was obtained in a concentration of 10^3 CFU/reaction in chicken meat samples .The reproducibility of the 691 bp fragment was approved by a repeated PCR assay. So PCR was the applied successfully in detecting this bacterium as the classical methods have some difficulties , since the incidence of non-pathogenic strains is higher than that of *L. monocytogenes* and all species demonstrates the same phenotype ***[44]***, there was also the occurrence of Listeria-like organisms on the selective medium, but were not Listeria species and this just be confirmed with PCR ***[45].***

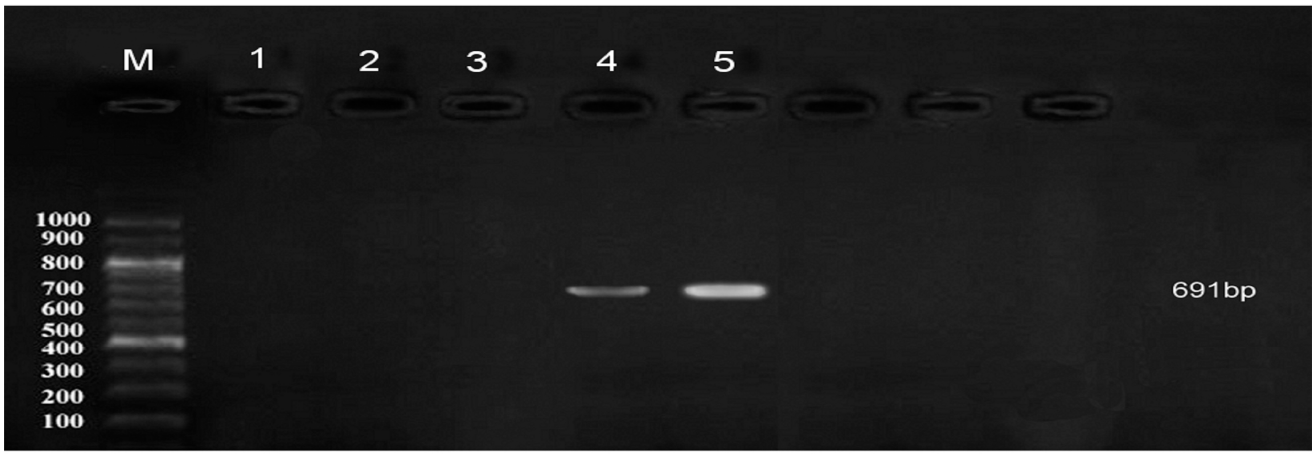

Figure 3 :Sensitivity of the PCR for the detection of *L.monocytogenese*, the first Lane represents DNA ladder 1 kb; Lanes 1 to 4: target gene isolated from 10, 10^2, 10^3, 10^4 CFU/ ml, respectively.

Conclusion

A rapid, and specific method was applied successfully for detect the virulence gene belonging to *Shigella flexneri, E.coli o157:H7* and *Listeria monocytogenese* from food samples. This particular typing method that based on PCR can be used for future surveillance studies and sensitive screening in monitoring cases of outbreaks. However use of gel electrophoresis end point detection have hampered the transition of these methods from research to routine use in food microbiology laboratories.

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