A RAPID METHODS FOR PCR BASED ON DETECTION OF *SALMONELA* SPP. AND *STAPHYLOCOCCUS AUREUS* IN SPIKED AND NATURALLY CONTAMINATED FOOD

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ABCTRACT

A rapid method for detection of food-borne pathogens, gene specific PCR, was used to detect two kinds of bacteria (*Salmonella spp. and Staphylococcus aureus)* from food. Cultures of artificially inoculated foods were spiked ,with reference bacteria at known concentrations and DNA was isolated from each food sample before and after enrichment, using phenol -chloroform based method, Positive results were obtained using gene specific PCR (targeting the *invA* gene in *Salmonella spp*. and *Sa 442* gene *in Staphylococcus aureus*) just after enrichment step, that produced specific amplicons of the expected sizes which was 284bp in *Salmonella spp*. and 108 bp in *Staphylococcus aureus*. The detection limit of the assay was 10^3 CFU/ ml for the two kinds of bacteria. No results were obtained using the same primers with five other types of bacterial strains which improve it s specifity. The same technique was used for detection of the two kinds of bacteria in some naturally contaminated foods. To achieve this, 13 food samples were collected from Sulaymani market during April-October, 2012 including meats (meat of beef, sheep, goats, fresh chicken, and frozen chicken) and vegetables (celery, tomato, cucumber, pepper, lettuce, broccoli, carrot, and leek) DNA isolated from the samples after two enrichment steps ,the results of PCR, indicate the detection of *Salmonella spp*., in three out of 13 food samples tested, whereas the detection of *Staphylococcus aureus* achieved in four out of 10 food samples tested. This study indicate that PCR is a good way for the rapid detection of *Salmonella spp* and *Staphylococcus aureus* in food.

Key words : Identification , gene specific PCR, food-borne pathogens.

طرائق سريعة بالاعتماد على تفاعلات التضاعف المتسلسلة للدنا للكشف عن ال *Salmonella spp* والـ *Staphylococcus aureus* الملوثة للغذاء

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المستخلص

لغرض ايجاد طريقة سريعة للكشف عن الاحياء المرضية الملوثة للغذاء تم استخدام التفاعل التضاعفي لسلسلة الدنا Polymerase Chain Reaction or PCR) والمتخصصة للنوع (Species specific PCR) وذلك للكشف عن نوعين من البكتريا المرضية وهما Salmonella spp و Staphylococcus aureus في الاغذية. لوثت نماذج من الاغذية بتراكيز معينة بالنوعين الملوثين المدروسين وتم تنشيطها، بعدها تم استخلاص الدنا من نماذج الغذاء الملوثة قبل وبعد التنشيط وتم الحصول على نتائج ايجابية بتطبيق تفاعلات ال PCR مستهدفة الجين *invA* في *Salmonella spp.* والجين Sa 442 في *Staphylococcus aureus* بعد التنشيط فقط وذلك بالحصول على قطع متضاعفة بحجم 284 زوج قاعدي في Salmonella spp وبحجم 108 زوج قاعدي في Staphylococcus aureus. ان اقل تركيز تم الكشف عنه بهذه التقانة هو 10^3 مستعمرة لكل مل و لكلا النوعين البكتريين المدروسين .تم التاكد من تخصص البادئات وذلك باستعمالها ضمن تفاعلات ال PCR مع خمسة انواع اخرى من البكتريا اذ لم تظهر اي نتيجة ايجابية مع الانواع الخمسة .تم تطبيق هذه التقانة للكشف عن النوعين المدروسين في الاغذية المتلوثة بشكل طبيعي بها ،اذ تم جمع 15 نموذج من الاغذية من اسواق السليمانية خلال نيسان الى تشرين الاول شملت اللحوم كلحم البقر ،الاغنام ،الماعز،الدجاج الطازج والمجمد وبعض الخضراوات التي شملت الطماطة ،الخيار ،الفلفل ،الخس ،البروكلي ،الجزر،الكراث والكرفس وتم استخلاص الدنا من نماذج الغذاء بعد مرحلتين من التنشيط اثبتت نتائج التفاعل التضاعفي لسلسلة الدنا الكشف عن *Salmonella spp* في ثلاثة نماذج من الغذاء من مجموع 15 نموذج والكشف عن ال *Staphylococcus aureus* في اربعة نماذج من مجموع 10 نماذج من الاغذية .وهذه الدراسة اثبتت ان التفاعل التضاعفي لسلسلة الدنا والمتخصصة للنوع هي طريقة جيدة في الكشف السريع عن الملوثات البكتيرية في الاغذية.

كلمات مفتاحية: تشخيص، الاحياء المرضية الملوثة للغذاء، PCR .

INTRODUCTION

Food borne illness represents one of the major public health problems. *Salmonella* spp. is one of the predominant bacteria species that cause public health problems and still the leading cause of food borne infections all over the world (1). Transmission of Salmonella to humans is usually by consumption of undercooked meat, poultry products and other cross-contaminated foods, such as vegetables that are eaten without cooking (2). *Staphylococcus aureus* also recognized as one of the major bacterial pathogens which cause food-poisoning cases, the main topic of interest for food safety is, its ability to produce a wide spectrum of thermostable enterotoxins, which cause acute gastroenteritis after food consumption (3). It produces a large variety of enterotoxins but 95% of poisoning outbreaks are caused by classical enterotoxins: A, B, C, D and E (4). *Staph. aureus* has been isolated from: meat, chicken, milk ,dairy and fish products (4). 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 bacterial contamination of food based on specific microbiological and biochemical tests after their isolation in differential and selective growth media, these conventional method that used for Salmonella detection is time consuming, requiring 4-6 days for confirming the results (5). Also require from 5-6 days for detecting *Staph. aureus* in food, besides ,it is laborious and time-consuming (6).To overcome this disadvantage, several rapid and sensitive methods for the detection of food born bacteria in foods have been developed to decrease the detection time ,among these, polymerase chain reaction (PCR)has distinct advantages, in term of specificity, sensitivity, rapidity and accuracy as many studies used PCR in order to establish it s limits for the detection of Salmonella in food (7,8). The presence of Salmonella using gene specific PCR targeting *invA* gene, was also determined in 50 commercial top sirloin beef samples (9). Using primer targeting the *invA* primers based PCR for detection of Salmonella is very important because it confirmed by biochemical and serological assay (10). However pre-PCR sample preparation protocol is needed, including a pre enrichment step followed by selective enrichment to conform the diagnostic accuracy,which was shown to be 100% compared to the traditional culture method. (11). Recently real-time PCR was developed and validated for more sensitivity of detection of Salmonella in food (12). Using traditional method for detection of *Staph. aureus* in food is also insufficient for the precise identification of *Staph. aureus* colonies (13), besides they are labor-intensive and time-consuming, So species-specific PCR for detection of *Staph.aureus* have been applied that targeting various virulence genes including 16S-23S rDNA spacer region (14), the genes encoding the enterotoxins (15) and the accessory gene regulator (*agr*), which has been shown to regulate the synthesis of many virulence factors during bacterial growth (16). Recently, and for more sensitivity, real time quantitative -PCR procedure were used which was establish for the routine detection and quantification of *Staphylococcus aureus* in different food matrices (17). As there are no previous data exists for application of PCR for detection of food borne pathogens in food in Kurdistan region of Iraq , this study aimed to develop and optimize a sensitive method based on the gene specific PCRs for the rapid and definite detection of *salmonella spp. and Staph. aureus* in food samples, which can help to develop effective prevention and control strategies.

MATERIALS AND METHODS

Samples collection and preparation: Thirteen food samples including chicken meat, meat and vegetables were purchased, randomly from local food stores in Sulaymani market in Kurdistan region of Iraq. Each sample was of 25 g aseptically removed and placed in a sterilized plastic bag and transported to the laboratory, food samples were homogenized following the standard methods (18).

Bacterial strains: The types of Bacterial used in this study *(Salmonella* typhimurium and *Staphylococcus aureus*) were obtained from the Medya Diagnostic Centre (MDC) in Erbil, Kurdistan region-Iraq. Bacterial strains were cultured for 24 h in 10 ml in 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 (19). For the study of the sensitivity of PCR, serial decimal dilutions of the two cultures in phosphate-buffered saline (PBS) were plated onto trypticase soy agar (TSA) for each bacterium. The plates were then incubated at 37 Cº for 48 hours before enumeration. One ml of each dilution was used to inoculate 25g of food sample. Then each inoculated sample was placed in 225 ml of TSBYE medium and homogenized using a stomacher at 90s. Those cultures were incubated for 6h at 36 ºC. After incubation, bacterial DNA was extracted from each culture.

DNA extraction: Ten ml from each samples including (chicken meat, meat and vegetable) were used for DNA extraction following the method developed by (20) as follows: the pellets of 24 hours broth media were re-suspended in 2 ml of TE buffer (500 M Tris-HCl [pH 8.0], 100 M NaCl, 1 M sodium citrate, 5 M 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 thus, release 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 isoamyl used 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**.**

PCR analysis The primers sequences which were provided by Bionerr Company (Kores) were as follows: F/ GTGAAATTATCGCCA CGTTCGGGCAA and R/TCATCGCACCGT CAAAGGAACC which amplify 284bp in *salmonella spp*. and F/AATCTTTGTCGGT ACACGATATTCACG and R/CGTAATGA GATT TCAGTAGATAATACAACA which amplify 108bp in *Staph. aures* . The reagents required for PCR reaction mixed about 20-µl 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) and template DNA (25-50ng)and primer (10 pmol). The program was as follow for salmonella spp : One cycle for 94 ºC for 2 min and thirty cycles: Step 1: 95 ºC for 5 min,Step 2: 65 ºC for 1 min, Step 3: 72 ºC for 2 min and One cycle of 72 ºC for 5 min (Final extension). The same program was used except the Annealing temperature *which was 55* ºC *using the Staphylococcus aureus* gene targeting primers. Then the products were running on 1.2% agarose gel electrophoresis and stained by Ehidium bromide for detection of the amplified fragments. In order to evaluate and verify the specificity of PCR protocol , each primer pair was tested by PCR on DNA templates prepared from a panel of five different bacterial isolates including *Salmonella* typhimurium 12022, *Shigella flexneri* 14028, *Listeria monocytogenese* 25923, *E.coli O 157:H7* and *Staphylococcus aureus* 11994).

Determination of detection sensitivity: To determine 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(19). 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.

Method used for detection of salmonella in naturally contaminated food: A modified method based on (21) was used to isolate Salmonella From meat samples (meat of beef, sheep, goats, fresh chicken and frozen chicken) and vegetables samples (celery, tomato, cucumber,pepper, lettuce, broccoli, carrot and leek). Twenty-five gram of each

samples were weighed and transferred into sterile Stomacher bags, each containing 225 ml of buffered peptone water (BPW) incubated at 37 ºC water bath with shaking at 100 rpm/min for 6 h, then 1 ml pre-enriched rinse was transferred to 10 ml tetrathionate (TT) broth and incubated at 42ºC with shaking at 100 rpm/min for 24 h. After incubation, the TT broth was used to extract DNA of Salmonella as mentioned above.

Method used for detection of *Staphylococcus aureus* in naturally contaminated food: Based on (22) *Staph.aureus* isolated from food samples including meats of (sheep ,fresh chicken, frozen chicken and beef) and vegetables including(lettuce, pepper, leek, tomato, celery ,cucumber) Twenty-five g of each sample were weighed and transferred into sterile Stomacher bags, containing 225 ml of Brain heart infusion broth in 37ºC water bath with shaking at 100 rpm/min for 6 h, and 1 ml pre-enriched rinse were transferred into to selective agar (Manitol salt agar) after incubation for 24 hours, this selective media was used to extract DNA of *Staph.aureus* as mentioned previously.

RESULT AND DISCUSSION

Detection of sensitivity. The results shown that total DNA extracted by a phenol-chloroform method provided a sufficient quantity of bacterial DNA to allow for a rapid and simple detection procedure. When evaluating PCR for the detection of microorganisms, two important criteria must be satisfied: specificity and sensitivity (23). In order to evaluate and verify the specificity of the primers in this study, they were tested by PCR on DNA templates prepared from the different bacterial isolates beside Salmonella *typhimurium* and *Staph.aureus* other strains including *:E. coli, Shigella flexneri*, and *L.monocytogene*se,. The analyses indicated that the primer pairs were specific for their corresponding target organisms, and the primers specifically amplified 284bp, 108bp, in bacterial strains belonging to *Salmonella spp.* and *Staphylococcus aureus*, it mean that they did not give any false positive results. These results were parallel to those obtained by other researchers (24),(25). To evaluate and verify the sensitivity of PCR for detection of *Salmonella* spp. in food samples, after they were spiked with their strains at four inoculums levels. *Salmonella typhimurium* were detected using gene specific PCR, the detection limit was 10^3CFU/ml (Fig. 1, Lane 3) ,Similar results have been reported previously by other studies (26, 27,28), who observed that at least 10^3 cells/ml ,must be present to give positive results by PCR. So this assay has the potential to become a standardized method for routine analysis of food for the presence of *Salmonella spp*. However using new methods of PCR techniques such as real time PCR methods recommended which allowed more sensitive detection of *Salmonella spp*. in food, but it is not available in our country till now.The same method used to verify the sensitivity of PCR for detection *Staph. aureus* **,** gene specific PCR applied targeted to the *Sa 442* gene on food samples artificially contaminated with *Staph. aureus*, at the levels of $10^1$to 10^4 CFU/ g .Amplified product (108 bp) obtained just in two of the samples (10^3,10^4 CFU/ ml) (Figure 2), This is in agreement with (30) who found that at least 10^3 CFU /g was needed to obtained positive results for *Staph.aurus* in food samples . On the other hand, two samples produced negative results (10,10^2 CFU/ ml),this indicate that the sensitivity of this technique is not enough for detecting them .it was frequently reported the variation in the sensitivity of PCR when it was applied to artificially inoculated food. One of the reasons is variation in the procedure used for the isolation of nucleic acids from the complexes food matrices.

Detection of *Salmonella spp*. in naturally contaminated food. The results of applying specific PCR assay, targeting the *invA* gene, combined with a two step enrichment for the detection of Salmonella shown in figure 3 ,which indicate the detection of *Salmonella spp*., by generating a PCR product of 284 bp in three from the 16 samples of food (18.75%), this is in agreement with the results of other (30) which showed that the rate of contamination of *Salmonella spp*. detected in un spiked foods was 8.67% ,also coincide with (31) who detected *Salmonella serovars* in 5.92% of examined poultry samples. The reason of using the primers that targeting the

invA gene in *salmonella* spp., is that the gene now has been recognized as an international standard for detection of the genus of Salmonella (23) ,it mean that all *Salmonella species* carry the *invA* gene, which is not carried by any other bacterial species (32). This gene encodes a protein in the inner membrane of bacteria which is responsible for invasion to the epithelial cells of the host (33). It is worth to consider that no amplified products were obtained in natural contaminated food, without enrichment steps, the same results obtained in other studies (34,35) this is because enrichment steps.

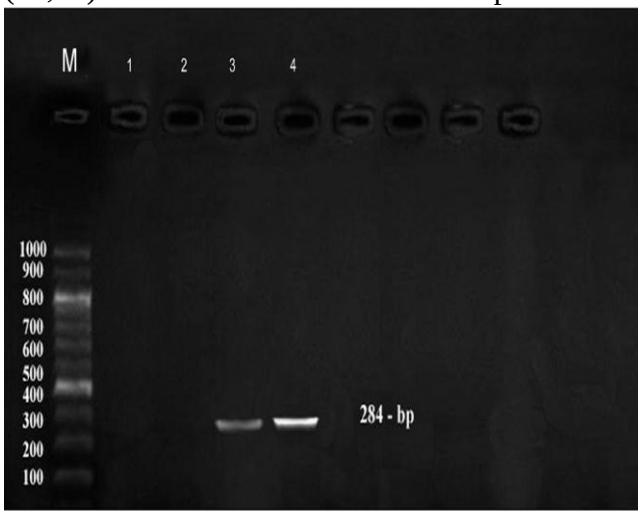

Fig 1: Sensitivity of the PCR for the detection of *Salmonella Typhimurium*, the first Lane represents DNA ladder 1kb; Lanes 1 through 4: target gene isolated from 10, 10^2, 10^3 and 10^4 CFU/ ml, respectively

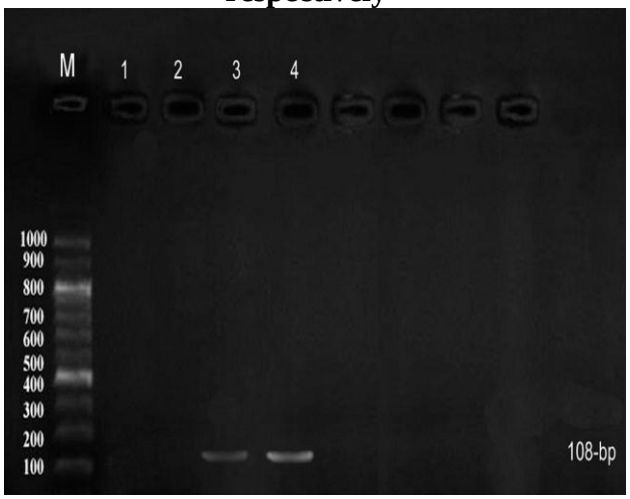

Fig 2: Sensitivity of the PCR for the detection of *Staphilococcuse aureus,* the first Lane represents DNA ladder 1 kb; Lanes 1 through 4: target gene isolated from 10, 10^2, 10^3 and 10^4 CFU/ ml, respectively

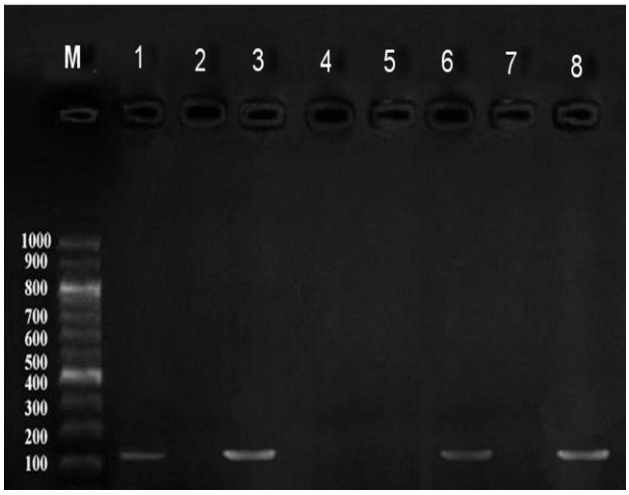

Fig 3: Electrophoretic analysis of 284 bp amplification product from *invA* gene of *Salmonella spp.* Isolated from some samples of food M: 1kb marker. *Lane* 1: represent chicken meat, *Lane* 4 represents tomato, *Lane 8* represents frozen chicken

increased the detection limit of these bacteria and diluted the Possible biological contaminants such as blood and fats present on meat samples which can cause PCR inhibition (34). So PCR offers a great and rapid diagnostic tool for *Salmonella spp*. detection and the 33h of this PCR assay offers a good tool in compared to the 3-5 days in using traditional methods for detection of Salmonella species in food samples.

Detection of *Staph. aureus* in naturally contaminated food :The efficacy of the developed PCR-based detection method was further evaluated for analysis of *Staph. aureus* in food samples using specific primers targeted the *Sa* 442 gene, and result obtained just after it combined with a two step enrichment , this is in agreement with the resnlls of other researchers 127, 387 (36,37). The results in figure 4 showed 108 bp amplified products in four out of ten food samples which were positive for *Staph. aureus* including :tomato, lettuce, fresh chicken meat, frozen chicken meat , this is in agreement with (38,22) who detect *Staph. aureus* by PCR in less than 50% of naturally contaminated foods, this mean that the developed PCR-based, method was very effective in *Staph.aureus* detection. Moreover, the method was able to overcome the problematic colony identification combined the traditional culture methods ,beside the

speed, sensitivity, and exactness of the obtained results

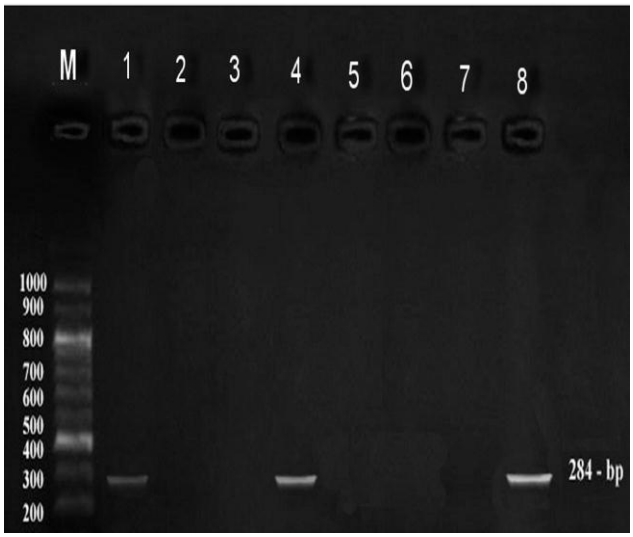

Figure 4: Electrophoretic analysis of 108 bp amplification product from *Sa442* gene of *Staph.aureus* isolated from some samples of food M: 1kb marker. *Lane* 1: represents tomato, *Lane* 3 represents frozen chicken, *Lane 6* represents lettuce and *Lane 8* represents chicken meat

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