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Development of rapid and simultaneous detection of four major foodborne pathogens using a multiplex PCR method

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Abstract

Food borne diseases are an important public health problem has major impacts on human health, also affect trade and economic issues. Developing microbial cultures to detect foodborne pathogens is time-consuming and expensive. The aim of this study is to develop a multiplex (mPCR) method for the simultaneous detection of *Staphylococcus aureus*, *Escherichia coli*, *Listeria monocytogenes* and *Salmonella enteritidis*. Buffered peptone water (BPW) was used as pre-enrichment. Simplex and multiplex PCR settings were optimized and applied to both pure co-cultures and artificially inoculated ready-to-eat food samples (falafel and chicken nugget). The four microorganisms could be detected individually and in enrichment media artificially inoculated at 10^1 CFU/mL by mPCR. In conclusion, the individual and combined growth of *E. coli*, *S. enterica*, *S. aureus* and, *L. monocytogenes* with low levels of contamination in the presence of food matrices such as falafel and chicken nuggets is effectively supported by BPW broth as co-culture medium before mPCR detection. The proposed protocol for pre-enrichment of *E. coli*, *S. enterica*, *S. aureus* and, *L. monocytogene*s in takes approximately 34 hours , compared to culture methods that require at least 7 days. This notably reduces analysis time, effort, and cost.

Keywords: Ready-to-Eat Food, multiplex PCR (mPCR), *Escherichia coli*, *Staphylococcus aureus*, *Salmonella enterica*, *Listeria monocytogenes*

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1. Introduction

Pathogenic bacteria isolated from food are taken as biological agents that cause more than 70% of food-borne diseases. It is estimated that more than 600 million individuals around the world suffer from food-borne diseases every year. Foodborne diseases are a critical public health problem that has major impacts on human wellbeing, and also affects financial and economic issues.

Hence, it seems necessary to develop a simple, fast, sensitive and cheap method to identify these bacteria (1). One of the complex challenges of food security is the development of industrialization and international trade of various food products (2). However,the consumption of a special diet such as the organic food or, consumption of raw or prepared food has caused an excessive increase in cases of food poisoning (3). Microbial contamination of food is a major healthcare problem. *Escherichia coli, Salmonella enterica*, *Staphylococcus aureus*, and *Listeria monocytogenes* are major pathogens that contribute to food poisoning (4). The current diagnostic methods are very complex and require pre-enrichment, enrichment and selective environments, and ultimately

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require biochemical or serological tests for definitive diagnosis (5-7). While these methods are very complicated, time-consuming, expensive and subject to human error, they are still considered the gold standard (8). Recently, the use of molecular methods such as polymerase chain reaction (PCR) has shown a lot in studies related to the outbreak of food-related epidemics, food contamination and analyses (9-11). One of the disadvantages of molecular methods is that the reliability and sensitivity of these methods depend on the number of bacteria in the sample. For example, if the number of bacteria in the food is low, it is difficult to detect the pathogen (5). As a result, to increase the efficiency of these methods, an enrichment step is required before extracting the genetic material to increase the number of pathogens (5, 12). So far, many studies have been conducted to simultaneously detect several pathogens in one reaction. Iun *et al*. developed a multiplex PCR method for screening and detecting of *Escherichia coli* O157: H7, *Listeria monocytogenes*, *Salmonella* spp, *Vibrio cholerae*, *V. parahaemolyticus*, and *Staphylococcus aureus* which are six common foodborne pathogens in Macao. The study results indicated that the m-PCR is a promising technique for the rapid detection of foodborne bacteria for routine monitoring and risk assessment of food sources (4). On the other hand, Chen *et al.* described a multiplex PCR technique for the identifying five food-relevant virulence pathogenicity genes of intestinal pathogens. Five pairs of primers have been designed primarily based on the nuc gene for *Staphylococcus aureus*, hlyA gene of *Listeria monocytogenes*, ipaH gene of *Shigella flexneri*, lysP gene of *Yersinia enterocolitica* and tpi gene of *Clostridium difficile*. The study suggested that this rapid method could serve as a reference for conducting food safety investigations and microbial epidemiology (13). This study aims to develop a multiplex PCR method for the simultaneous detection of *Staphylococcus aureus*, *Escherichia coli*, *Listeria monocytogenes* and *Salmonella enteritidis* to save time, reduce costs, make work easier, and reduce human errors.

2. Material and methods

2.1. Used Bacterial Strains

The four most significant pathogenic microbes in nourishment were examined. Strains used: *E. coli* ATCC 101, *S. enterica* ATCC 4266, *L. monocytogenes* ATCC 936, and *S. aureus* ATCC 435, were obtained from the variety collection of the Pasteur Institute (ATCC, Tehran, Iran). To evaluate the specificity of mPCR, strains from different roots were used: *Listeria innocua* (ATCC19115), *Bacillus cereus* (ATCC6633) and *Listeria grayi* (ATCC700545)

Following a separate culture of each bacterial strain, the plates were incubated for 24 h at 37 ºC using the plate counting agar streak method (PCA, Scharlau, Spanish).

2.2. Co-Culture Medium Selection and Assessment of Recovery Capacity

Based on the study of Boukharouba *et al*., peptone water buffer medium (BPW Scharlau, Spanish) was chosen as the enrichment medium in this study (14).

2.3. Preparation of the Inoculum

In order to create fresh pure cultures, an isolated colony of each target bacterium was inoculated separately into 10 ml of BPW. The colonies were then incubated for 24 hours at 37 °C while stirring at 150 rpm. The UNE-EN ISO 7218:2008 standard was used to determine the final concentration, was in the range of 10^8–10^9 CFU/ml.

2.4. Effect of BPW on Growth of the Individual and Co-Culture

In order to assess BPW's ability to recover from low initial inoculum concentrations in individual pure cultures, three initial inoculums concentrations of 10^3, 10^2, and 10^1 CFU/mL were tested for each target pathogen in a final volume of 10 ml. In each of the three experiments (Experiment I: 10^3 CFU/mL; Experiment II: 10^2 CFU/mL; and Experiment III: 10^1 CFU/mL) had the same concentration of the four target bacteria. One milliliter aliquot of each obtained culture was frozen at -20 °C for PCR analysis. All plates were incubated in BPW at 37 ºC for 24 hours. The number of CFU/mL was then calculated using the formula given in UNE-EN ISO 7218:2008.

2.5. Effect of BPW on the growth of co-cultures from an artificially infected food matrix

Two types of ready-to-eat foods were tested, namely falafel and chicken nuggets, which

were purchased from local markets and used fresh without any disinfection treatment to preserve background microbiota products. This was done in order to assess the efficiency of BPW recovery when co-cultured from artificially inoculated foods with background microbiota. To prepare food matrix (falafel and chicken nuggets), first 10 grams of food was dissolved in peptone culture medium and then dilutions were prepared from this initial stock. After mixing for five minutes, the mixtures were incubated at 37 ºC for 24 h.

2.6. DNA Template Preparation (Thermal Lysis Method)

After centrifugation of 1 mL of the collected aliquots at 14,000 rpm for 10 minutes, the pellet was resuspended in 100 µL of sterile Milli-Q water. Then the suspension was stirred by shaking, boiled at 100 °C for 10 minutes and quickly cooled on ice for 5 minutes. After centrifugation at 12,000 rpm for 5 minutes, the supernatant was collected and kept at -20 °C until used as a template for PCR (15).

2.7. Primers

Primer GADA 670-F/R for the detection of *E. coli*, Nuc 484-F/R primer for the detection of *S. aureus*, LM 404-F/R primer for the detection of *L. monocytogenes* and SalinvA 284-139/141 primer for the detection of *S. enterica*. The genes targeted to the coding gene were selected from previous studies (16, 17). The specificity of each primer was checked *in silico* by using the Primer Blast program, http: //blast.ncbi.nlm.nih.gov/Blast.cgi. Multiplex PCR was performed using a combination of four specific primer pairs in one reaction (Table 1).

2.8. Simplex PCR

The same simplex PCR procedures were used to test each primer. These conditions were achieved after optimization tests that included an annealing temperature gradient (56, 58, and 60 ºC) for each simplex PCR (Applied Biosystem 9700, California, USA). PCR was performed according to the following protocol: three minutes of initial denaturation at 94 °C, 35 cycles (denaturation at 94 °C for 30 s, annealing at 58 °C for 30 seconds, extension at 72 °C for 60 seconds, and final extension at 72 °C for 7 minutes). After amplification, 5 µL of each PCR product was mixed with 1 µL KBC power load and electrophoresed (Bio-Rad Co. USA) for 50 min at 100 V in a 1.2% agarose gel.

2.9.Multiplex PCR (mPCR)

mPCR was designed to simultaneous detect of *S. aureus*, *E. coli*, *S. enterica* and *L. monocytogenes* in a single reaction. After multiplexing, two tests were optimized: one to measure the changes in the number of cycles (30, 35, and 40 cycles) and the other to measure the annealing temperature gradient (56, 58, and 60 ºC). A total volume of 20 µL contains 10 µl of taq 2× Mastermix RED (1.5 mM $Mgcl_2$), 100 ng extracted DNA, and forward and reverse primers in final concentrations of 250 nM each, except for *S. aureus* 500 nM set as the best conditions. The final primer concentrations were optimized experimentally. After the reaction was completed, 5 µL of each PCR product was mixed with 1 µL KBC power load and electrophoresed at 100 V for 50 min in a 1.2% agarose gel (1).

2.10. Assessment of Specificity

The specificity of the mPCR was assessed

Table 1. Primer used .

Strains	Primers	Sequences	Size	(ref)
E. coli	GADA/F	5′-ACCTGCGTTGCGTAAATA-3′	670 bp	(14)
	GADA/R	5′-GGGCGGGAGAAGTTGATG-3′		
S. aureus	Nuc/F	5′-CTTTAGCCAAGCCTTGACGAAC-3′	484 pb	(15)
	Nuc/R	5′-AAAGGGCAATACGCAAAGAGGT-3′		
L. monocytogenes	LM404/F	5′-ATCATCGACGGCAACCTCGGAGAC-3′	404 bp	(15)
	LM404/R	5′-CACCATTCCCAAGCTAAACCAGTGC-3′		
S. enterica	SalinvA139	5′-GTGAAATTATCGCCACGTTCGGGCAA-3′	284 bp	(14)
	SalinvA141	5′-TCATCGCACCGTCAAAGGAACC-3′		

by distinguishing between target and non-target bacteria. The selectivity of each primer was tested by mixing the four primer pairs with various random combinations of positive control DNA and then amplifying using the optimized multiplex PCR conditions mentioned above. This allowed for the detection of any cross-hybridization that could result in mispriming between the four primers and the four target DNAs. DNA was extracted from pure *E. coli* ATCC 25922, S. aureus ATCC 6530, L. monocytogenes CECT 936 and *S. enterica* NTCC 3890 as positive controls.

Then, *Enterococcus faecalis* (NTCT 8213), *Lactobacillus acidophilus* (ATCC 8001), *Enterobacter cloacae* (PTCC 1237), *Bifidiobacter* spp were then used to assess the specificity of the mPCR.

2.11. Assessment of Sensitivity

To determine the detection limits of each simplex and mPCR assay, the sensitivity was first assessed using DNA extracted individually from the four pure cultures in BPW. Experiments were then performed with DNA isolated from co-cultures established at BPW, both with and without artificial inoculation of the food matrix, as described in Section 2.2.

2.12. Positive and negative predictive value

The index referred to is actually the positive predictive value, which denotes the ratio of truly positive samples to all cases identified as positive by the test. The negative predictive value, on the other hand, represents the ratio of truly negative samples to all cases labeled as negative by the test. To assess these indices, a PCR test was initially conducted on 30 distinct food samples intentionally contaminated, as well as 30 uncontaminated samples. Subsequently, utilizing the provided formulas, the positive and negative predictive values were computed (18).

$$positive\ predictive\ value = \frac{true\ positive}{true\ positive + false\ positive}$$

$$negative\ predictive\ value = \frac{true\ negative}{true\ negative + false\ negative}$$

2.13. Limits of detection when isolating BPW from a co-culture

Following the optimized conditions above, mPCR was performed on DNA extracted from 1 mL of each co-culture containing the 4 target microorganisms in artificially inoculated food matrices (falafel and chicken nugget), initially inoculated at level 10^3; 10^2; 10^1 CFU/mL.

3. Results

3.1. Effect of BPW on growth of individual bacteria

In individual pure cultures, evaluation of BPW recovery capacity from low initial inocula revealed relatively similar and stable rates, with 10^8 CFU/mL for *E. coli*, *S. enterica*, and exceeded 10^7 about *S. aureus* and, *L. monocytogenes*.

3.2. Effect of BPW co-culture on recovery

Recovery rates from chicken nugget samples were 10^7 CFU/mL for the Gram-negative bacteria and 10^6 CFU/mL for the Gram-positive bacteria based on co-cultivation in BPW broth from a food matrix artificially supplemented with 10^3 CFU/mL of each target bacteria. It showed a relatively stable rate close to the growth of individual cultures. It was over 8.3×10^8 CFU/mL for *E. coli*, 7.4×10^7 CFU/mL for *S. enterica*, 5.8×10^6 CFU/mL for *S. aureus* and more than 6.4×10^6 CFU/mL for *L. monocytogenes*.

3.3. Simplex PCR specific amplification

The effectiveness of the DNA extraction process and the proper functioning of each primer pair was evaluated using simplex PCR detection from individual cultures. Results showed specific amplification of *E. coli*, *S. enterica*. *S. aureus*, *L. monocytogenes*, and Generated amplicons were with different sizes of 670 bp, 284 bp, 484 bp, and 404 bp, respectively. These bands appeared as separate bands on the electrophoretic gel and did not generate any nonspecific products (figure 1).

3.4. Mutiplex PCR

After *in silico* validation and simplex PCR, primers were multiplexed by progressive integration in multiple duplex and triplex PCR reactions. The 4 primer pairs were then combined with the corresponding target DNA in a single reaction PCR. Conditions such as annealing temperature and concentration balance of the four primers in the reaction were optimized to ensure amplification of the four target fragments and prevent nonspecific reactions. Simplex, duplex, triplex, and quaduplex PCR provide accurate detection and do

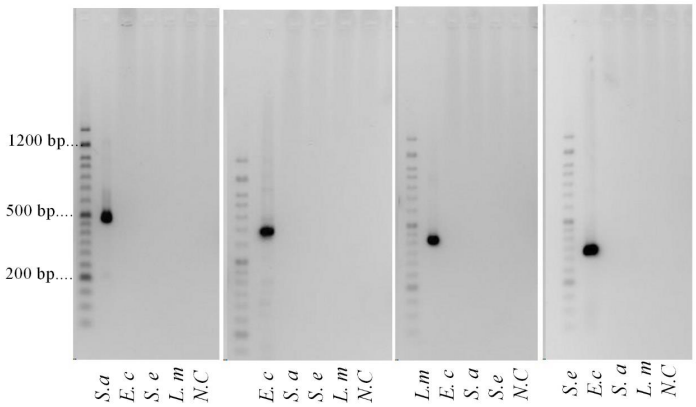

Figure 1. Detection Specificity of *E. coli* (E.c), *S. aureus* (S.a), *L. monocytogenes* (L.m), and *S. enterica* (S.e) primers and negative control (N.C) in simplex PCR.

not show nonspecific products on electrophoretic gels (figure 2).

3.5. Specificity evaluation

Consideration of evaluation of specificity of multiplex detection of DNA extracted from reference strains (*E. coli* ATCC 25922, *S. aureus* ATCC 6530, *L. monocytogenes* CECT 936, and *S. enterica* NTCC 3890). Each primer amplifies only its own target gene. Similar to other reference strains, *Enterococcus faecalis* (NTCT 8213) , *Lactobacillus acidophilus* (ATCC 8001), *Enterobacter cloacae* (PTCC 1237), *Bifidiobacter* spp no detection was observed, confirming that each primer was completely selective for its target gene.

3.6. Sensitivity and Detection Limits evaluation

mPCR was done on DNA extracted from BPW co-cultures in the presence of food matrices artificially inoculated with 10^3; 10^2; 10^1 CFU/mL of each target bacteria in the presence of background microbiota. The mPCR was able to detect all bacteria in matrices artificially inoculated with as much as 10^1 CFU/mL of each target pathogen after incubation without any nonspecific products (Figure 3).

3.7. Positive and negative predictive value

Based on the provided information, the calculations for the PPV and NPV appear to be as follows: Given that there are 33 positive cases reported, the PPV rate is calculated as 91%. The NPV is calculated as 100% because all the cases reported as negative by the test are truly healthy. This indicates that there are zero false negative cases, and the test's ability to correctly identify healthy samples is perfect.

4. Discussion

The goal of this study was to improve a

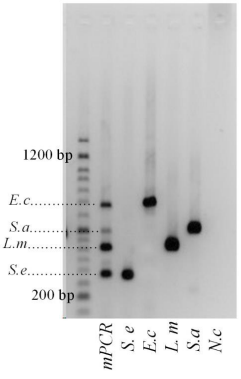

Figure 2. mPCR detection with mix DNA of *E. coli* (E.c), *S. aureus* (S.a), *L. monocytogenes* (L.m), and *S. enterica* (S.e) primers and negative control (N.C) and Detection sensitivity of all target in multiplex PCR.

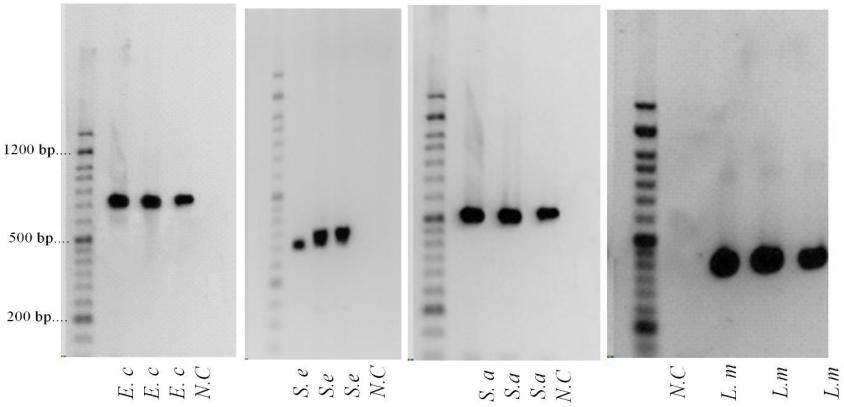

Figure 3. Detection sensitivity of *E. coli* (E.c), *S. aureus* (S.a), *L. monocytogenes* (L.m), and *S. enterica* (S.e) and negative control (N.C), in multiplex PCR inoculated with 10^3; 10^2; 10^1 CFU/mL of each target bacteria.

rapid and simple method for the simultaneous detection of 4 microorganisms: *E. coli*, *S. enterica*, *S. aureus*, and *L. monocytogenes*, from ready-to-eat foods in comparison to expensive and time-consuming techniques that are available in most laboratories.

We also aimed to develop a protocol that includes a 24 h co-culture step in a BPW broth along with the standard mPCR detection of semi-cooked ready-to-eat food products. Our objective in this investigation was to pioneer the development of a multiplex PCR-based diagnostic kit for ready-to-eat food in Iran.

Previous studies have established simplex or multiplex PCR assay techniques for the simultaneous detection of some foodborne pathogens in seafood, strawberry, blackberry, lettuce, milk and meat (1, 14, 19). Our mPCR construction is based on a combination of four highly selective primers cited in several previous studies and has been optimized to ensure the most sensitive detection possible in the simultaneous detection of artificially inoculated semi-cooked foods. The current sensitivity and recovery results in our study were similar to those previously reported in raw foods (14). In addition, the enrichment step before DNA extraction improves the detection limit of the PCR and avoids false negative outcomes.

BPW medium was chosen because it is commonly used as a preamplifier broth in many ISO protocols and has a high ability to elute bacteria from ready-to-eat foods. Because it is a common and rich medium, it supports the growth of all four microorganisms to the extent that they can be detected by PCR. Previous studies have shown that BPW medium is highly effective in detecting *E. coli*, *Salmonella* spp, *S. aureus* and *L. monocytogenes* after 16 h of growth in BPW from an artificially inoculated samples without problematic interference from the background microbiota (20, 21).

To assess the recovery ability, individual and co-cultures in BPW broth were performed at initial inoculum doses of 10^3, 10^2, and 10^1 CFU/mL. The highest density was achieved with BPW after a 24-hour incubation period in individual cultures.

Furthermore, we found that the initial growth inoculum levels of all target microorganisms were slightly reduced. However, competition effects appear to have a significant impact on the resulting recovery rates. Gram-negative bacteria appeared to be least affected, while growth was more decreased in Gram-positive bacteria. Previously, this competitive growth effect was mentioned during co-culture (22).

Although the recovery of these microorganisms in the BPW co-culture appears to be less efficient compared to the rate of individual culture, the resulting growth is more than sufficient for mPCR detection. Both falafel and chicken nugget matrices tested were selected as ready-to-eat food models because they can be consumed without any cooking process. May be a carrier of bacterial strains that produce Shiga toxin (23).

The results of individual PCR and mPCR

assays are reported as highly specific and reliable primers to target the studied bacteria. Furthermore, selectivity was confirmed by the lack of amplification of non-target bacteria tested.

Co-culture with BPW broth inoculum (10^3, 10^2, 10^1 CFU/ml) successfully amplify bacterial DNA fragments up to 10 CFU/ml of the initial inoculum for mPCR-based detection

Furthermore, co-cultivation in the presence of food matrices artificially inoculated with each target microorganism (10^3, 10^2, 10^1 CFU/ml) resulted in very clear mPCR, despite the presence of background microbiota which is similar to the studies conducted in other countries and matrices (14, 24).

5. Conclusion

Individual and combined growth of *E. coli*, *S. enterica*, *S. aureus* and, *L. monocytogenes* with low levels of contamination in the presence of food matrices such as falafel and chicken nuggets is effectively supported by BPW broth as a co-culture medium before mPCR detection. The proposed protocol for pre-enrichment of *E. coli*, *S. enterica*, *S. aureus* and, *L. monocytogenes* in takes approximately 34 hours, compared to culture methods that require at least 7 days. This significantly reduces analysis time, effort, and cost.

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Conflict of Interest

The authors declare no conflict of interest.

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