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Rapid detection of bacterial food-borne pathogens by using multiplex PCR

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Rapid detection of pathogens in food becomes a critical and important demand for human safety, since most food-borne illnesses and deaths are caused by pathogenic bacteria. So application of rapid, sensitive method to detect food-borne pathogen is essential in controlling food safety .In this study, a two multiplex polymerase chain reaction (mPCR) technique for the simultaneous detection of these food-borne pathogens (*Salmonella ,Staph aureus, Bacillus cereus*, *Listeria monocytogenes, E. coli* and *Campylobacter spp.*) was done in culture broth and artificial food matrix. Pathogen-specific DNA sequences in the *inv*A, *clfA, groEL*, *16S rRNA, phoA, 23S rRNA* and genes were used as targets to design primers for the identification of *Salmonella*, *S. aureus*, *Bacillus cereus, Listeria monocytogenes, E. coli and Campylobacter spp.* respectively. The detection of sensitivity in this assay was 10CFU/ml of each pathogen in a culture broth and artificially inoculated samples after enrichment for 24 h. The mPCR assay proposed here can gain results within 24 h and correspond to the results obtained by the classical cultivation based on ISO methods, which will be valuable for food safety investigations.

Keywords: mPCR-food-borne pathogens- artificially inoculated

INTRODUCTION

Food safety is increasingly becoming an important global health concern. Food is one of the major sources of microbial pathogens in the developing countries. According to report from Centers for Diseases Control and Prevention (CDC), approximately 48 million people in the United States get sick, 128,000 people are hospitalized and 3000 people die annually due to food- borne diseases (Oliver et al., 2005; Centers for Disease Control and Prevention, 2011).

The common food-borne pathogens which are responsible for most of the food borne disease outbreaks were *Escherichia coli* O157:H7*, Staphylococcus aureus, Salmonella enteric, Bacillus cereus ,Vibrio spp., Campylobacter jejuni, Clostridium perfringens*, and shiga toxin-producing *Escherichia coli* (STEC) (Oliver et al., 2005; Scallan et al., 2011;Zhao et al., 2014).

Many outbreaks also caused serious economic loss for both consumer and industry. In Taiwan, during 1995 to 2001, 1,171 outbreaks of food-borne illness, including 109,884 cases, were reported in northern Taiwan, of which 735 (62.8%) were caused by bacterial infection. Bacterial pathogens, particularly *V. parahaemolyticus* (86.0%), *S. aureus*(7.6%), and *Salmonella* spp. (4.9%) (Su et al., 2005), *Bacillus cereus* (41.2%) was the main etiologic agents in central Taiwan. These outbreaks were mainly caused by mishandling of food at home (41.2%) and in school (34.3%) (Chang et al., 2003).

Salmonellosis is commonly associated with poultry (Salem et al., 2018) and eggs, along with

meat, unpasteurized milk or juice, cheese, spices and nuts, through consumption of food contaminated with *Salmonella* (Nowak et al., 2007; Modzelewska-Kapituła and Maj-Sobotka,2014; Zhou et al., 2014).In the United States, 1.4 million illnesses caused by *Salmonella* occur annually (Mead et al., 1999).

B. cereus is a common food poisoning bacterium with a great important role in the food industry since it produces heat resistant spores. In many certain strains *of B. cereus* a toxin cereulide produce that can cause lethal food poisoning (Granum,1994).

S. aureus could cause staphylococcal food poisoning through the ingestion of contaminated food with the enterotoxin produced by enterotoxigenic *S.aureus* from food (Asao et al., 2003).

Campylobacter jejuni is one of the most zoonotic pathogens between animal and humans in Egypt. Human illness due to *C. jejuni* infection is closely associated with consumption of poultry products (Barakat et al., 2015; Jakeen et al., 2015).

Listeriosis is a food-borne disease caused by food contaminated with the *Listeria monocytogenes (L. monocytogenes)* bacterium. *L. monocytogenes* is found in soil, vegetation and water. There are six species of Listeria, but only *L. Monocytogenes* causes disease in humans. It is a relatively rare disease, with 0.1 - 10 cases per million people per year, depending on the country or region of the world (Manganye et al., 2018).

Currently, the isolation approaches for detecting food borne pathogens are mainly focused on conventional culture-based assay (Lee et al., 2015) and PCR assays (Germini et al., 2009;Kawasaki et al., 2009; Zhang et al., 2015).

Although conventional culture method is recognized as 'gold standard' for the detection of food borne pathogens, it is laborious , tedious and time-consuming (2–3 days), including bacterial selective culture, biochemical reactions, serotyping and phenotypic assay (Karami et al., 2011; Park et al., 2011; Xu et al., 2012; Zhao et al., 2014; Lee *et al*., 2015;).Also it may be limited due to poor sensitivity, or slow-growing or poorly viable organisms in cases of low-level contamination(Lee et al., 2014).

Multiplex PCR assay is a powerful diagnostic tool, a low cost-effective assay that provides a rapid and high-throughput detection and screening of multiple targets (Xu et al., 2012).

When food samples were tested, pre-enrichment step of 10 h prior to the mPCR assay achieve a workable sensitivity (Babu et al., 2013). Sensitivity is important because a single pathogen present in food has the risk to cause infection.

The objective of this study was develop and standardization of a rapid, specific, and sensitive simultaneous detection of food borne pathogens using mPCR.

MATERIALS AND METHODS

Bacterial strains:

Salmonella Typhimurium (ATCC® 14028™), *Bacillus cereus* (ATCC® 0876™), *Staphylococcus aureus* (ATCC® 25923™), *Listeria monocytogenes* (NCINB® 50007), *E*. *coli* (ATCC® 25922) and *Campylobacter* (ATCC® 29428) were chosen as a model for food-borne pathogens in this study. These reference strains were purchased from the TCS Biosciences - Microbiological Supplies.

Pure cultures of strains were grown over night at 37^{o}c in buffered peptone water (BPW), then re-cultured and re-isolation to check them.

Plate count method was used to measure the colony- forming unit (CFU) of each bacterium

The used oligonucleotides

The DNA sequences, target genes, and amplicon sizes of the three pairs of primers used in this study are shown in Table 1.

Determination of sensitivity of the PCR protocol.

The sensitivity of the multiplex PCR procedure for the pathogens was studied using a 10-fold serial dilutions of pure cultures suspended in phosphate-buffered saline. Since cultured the detection limit of the reaction for the pathogens was found to be 10 bacterial cells.

Preparation of artificially contaminated ground beef.

Fresh ground beef was purchased from a local grocery store and determined to be free of tested pathogens by exposed to thermal and then examined standard cultural ISO recommended methods. One milliliter of each dilution of fresh overnight cultures was pour plated in plate count agar (Difco, Becton Dickinson), and colonies were counted after incubation at 37^{o}C for 24 h.

Preparation of artificially contaminated ground beef was performed by transferee one gram of ground beef to a sterile sample bag and inoculated with 1 ml of appropriate dilutions of

each target bacterial strain, then incubated at 37 ^{o}C overnight.

Table 1: primers sequences with amplified product.

Strains	Primers	Sequence	Amplified product	Reference
Salmonella	*invA*	GTGAAATTATCGCCACGTTCGGGCAA	284 bp	(Oliveira et al., 2003)
		TCATCGCACCGTCAAAGGAACC		
S. aureus	*clfA*	GCAAAATCCAGCACAACAGGAAACGA	638 bp	(Mason et al., 2001)
		CTTGATCTCCAGCCATAATTGGTGG		
Bacillus cereus	*groEL*	TGCAACTGTATTAGCACAAGC T	533 bp	(Daset al., 2013)
		TACCACGAAGTTTGTTCACTACT		
L. monocytogenes	*16S rRNA*	ggACCgggg CTA ATA CCg AAT gAT AA	1200 bp	(Kumar et al.,2015)
		TTC ATgTAggCgAgTTgCAgC CTA		
E. coli	*phoA*	CGATTCTGGAAATGGCAAAAG	720 bp	(Hu et al., 2011)
		CGTGATCAGCGGTGACTATGAC		
Campylobacter	*23S rRNA*	TATACCGGTAAGGAGTGCTGGAG	650 bp	(Wang et al., 2002)
		ATCAATTAACCTTCGAGCACCG		

Table 2: PCR condition

Primary denaturation	Secondary denaturation	Annealing	Extension	No. of cycles	Final extension
94˚C 5 min.	94˚C 30 sec.	55˚C 40 sec.	72˚C 45 sec.	35	72˚C 10 min.

Detection of food borne pathogen:

After 24 h incubation, 1 ml aliquot was collected from each dilution for DNA extraction in parallel the same sample was examined by traditional standard method.

Extraction of DNA

According to QIAamp DNA mini kit instructions, Catalogue no.51304

The QIAamp DNA Mini Kit provides silica-membrane-based nucleic acid purification from different types of samples. The spin-column procedure does not require mechanical homogenization, so total hands-on preparation time is only 20 minutes.

PCR conditions

After DNA extraction, Preparation of multiplex PCR Master Mix according to Emerald Amp GT PCR master mix (Takara) Code No. RR310A kit

Then mix carried out to programmable Thermocycler, Temperature and time conditions during PCR as following (in Table 2). Then PCR products were analyzed by Agarose gel electrophoreses.

RESULTS

Evaluation of the multiplex PCR assay with retail food samples:

Fifty samples (20 milk and milk products, 20 chicken and chicken products and 10 meat and meat products) collected from different sources and turned to bacteriology laboratory for isolation and identification of associated bacteria by classical methods and molecular techniques (Table 3).

First multiplex reaction:

Included the 3 pathogens: *Salmonella, S. aureus* and *Bacillus cereus.*

Second multiplex reaction:

Include the 3 pathogens *E. coli, Listeria monocytogenes* and *Campylobacter* spp*.*

Development of the specific multiplex PCR assay:

The obtained multiplex PCR products were 284 bp for *invA* for *Salmonella* spp., 638 bp for *clfA* for *S. aureus* and 533 bp for *groEL* for *Bacillus cereus* , the second multiplex PCR products were 1200 bp for *16S rRNA*for *L. monocytogenes* ,720 bp for *phoA* for *E. coli* and 650 bp for *23S rRNA* for *Campylobacter* spp*.*

Sensitivity of the multiplex PCR assay

The sensitivity evaluation of the PCR assay was carried out using a series of DNA sequence of the six pathogens in 10 folds dilution (from 10^9 to 10^1 CFU/ml).

There was a qualitative decrease in the intensity of the amplicons with the decrease of the

DNA concentration.

Table 3: occurrence of food pathogens from retail food samples using Multiplex Polymerase Chain Reaction (PCR) and Conventional culture methods:

Samples	The detected pathogens											
	Salmonella		*S. aureus*		*Bacillus cereus*		*E. coli*		*Listeria monocytogenes*		*Campylobacter* Spp.	
	Culture	PCR	Culture	PCR	Culture	PCR	Culture	PCR	Culture	PCR	Culture	PCR
Milk and milk products (n=20)	-	4	5	5	1	1	4	7	-	-	-	1
Chicken and chicken products (n=20)	-	8	3	5	1	1	2	4	-	-	-	-
Meat and meat products (n=10)	-	2	2	2	-	1	-	-	-	-	-	-
Total (n=50)	0	14	10	12	2	3	6	11	0	0	0	1
%	0%	28%	20%	24%	4%	6%	12%	22%	0%	0%	0%	2%

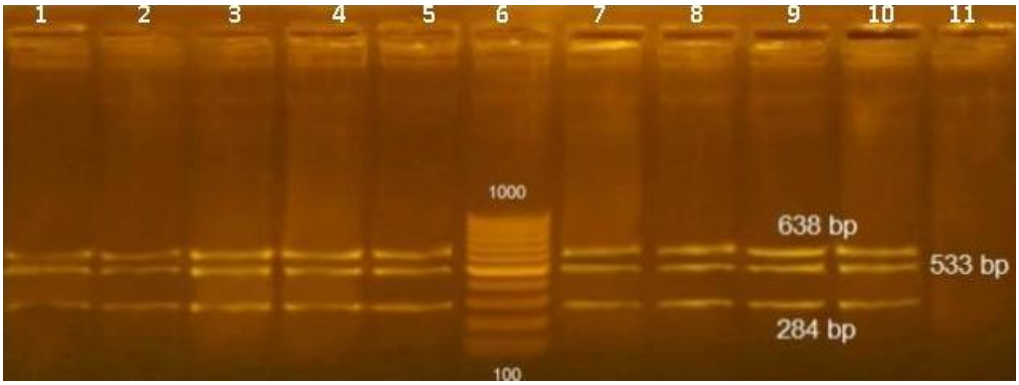

Figure 1.The multiplex PCR assay for detection of *Salmonella, Bacillus cereus* and S*taphylococcus aureus* in broth with different concentrations (10^9- 10^1 colony forming unit per ml) for the 3 pathogens mixture. Lanes 1, 2, 3, 5, 7, 8, 9 and 10: show the dilution of samples from 10^1 -10^9, Lane 6: molecular mass DNA marker (100bp) and Lane 11: negative control for the 3 pathogens.

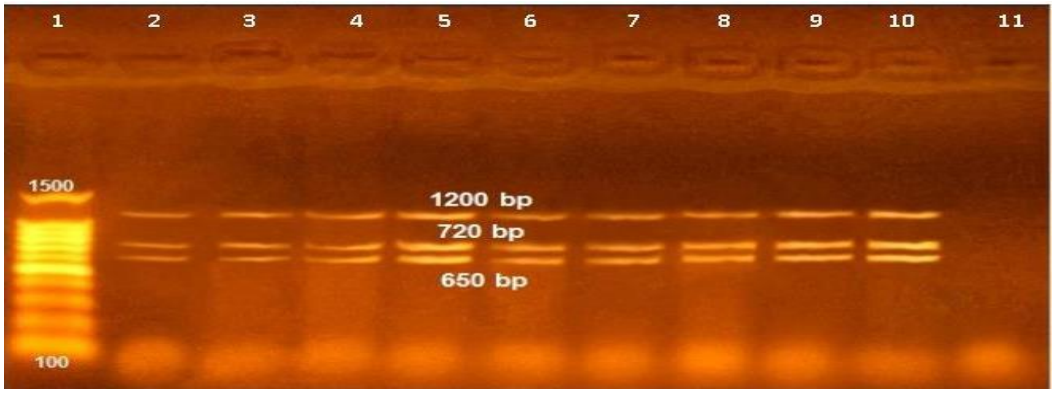

Figure 2.The multiplex PCR assay for detection of *Listeria monocytogenes, E. coli* and *Campylobacter* in broth with different concentrations (10^9- 10^1 colony forming unit per ml) for the 3 pathogens mixture. Lanes 2, 3,4, 5, 7, 8, 9 and 10: show the dilution of samples from 10^1 -10^9, Lane 1: molecular mass DNA marker(100bp), and Lane 11: negative control for the 3 pathogen

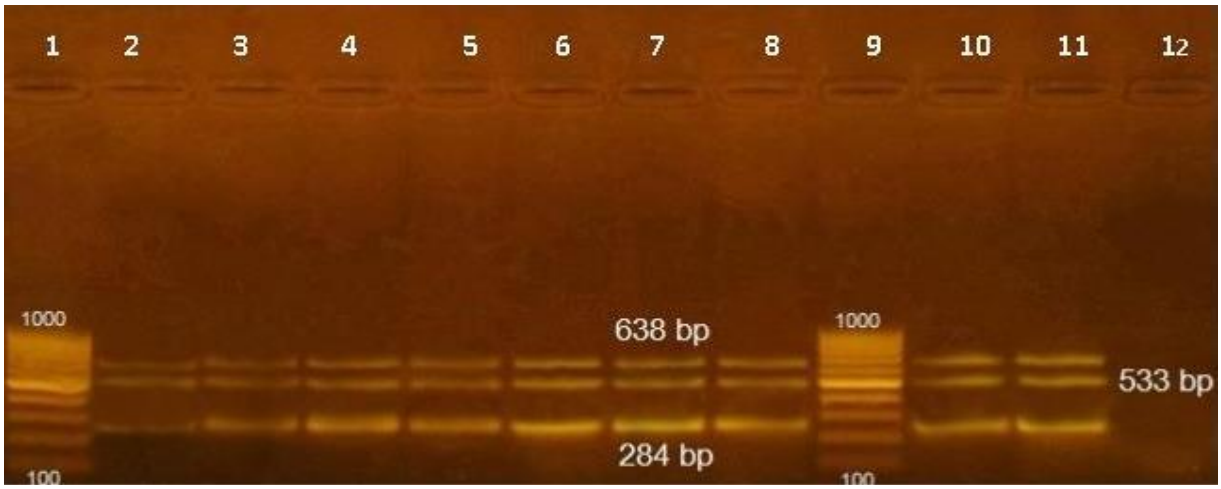

Figure 3.The multiplex PCR assay for detection of *Salmonella, Bacillus cereus* and S*taphylococcus aureus* in spiked meat with different concentrations (10^9- 10^1 colony forming unit per ml) for the 3 pathogens mixture. Lanes 2, 3,4, 5,6, 7, 8, 10 and 11: show the dilution of samples from 10^1 -10^9 , Lane 1,9: molecular mass DNA marker (100bp) and Lane 12: negative control for the 3 pathogens.

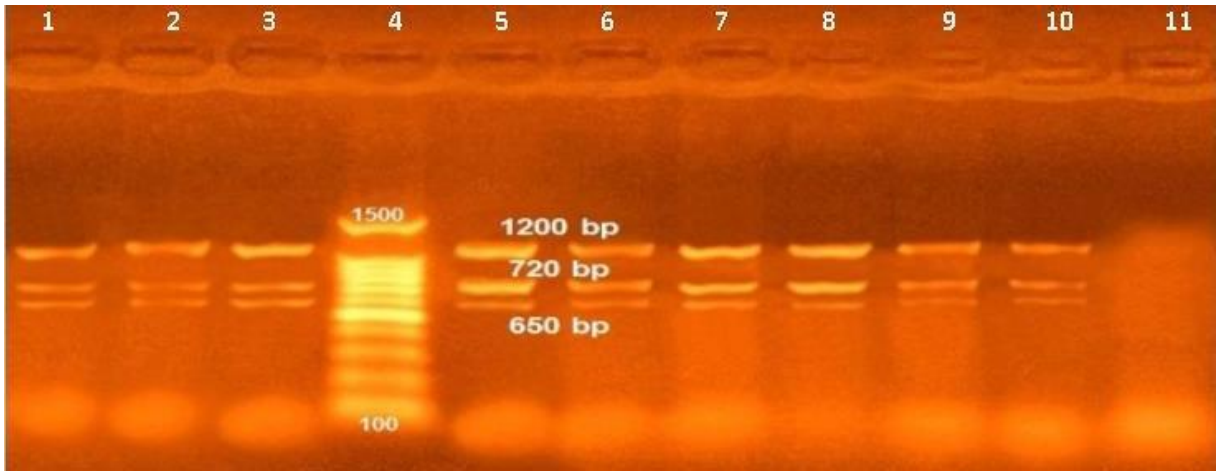

Figure 4.Application of the multiplex PCR assay for detection of target pathogens (*Listeria monocytogenes*, *E. coli* and *Campylobacter*) in spiked meat with different concentrations (10^9- 10^1 colony forming unit per ml)for 3 pathogens mixture. Lane 1,2,3,5,6,7,8, 9 and 10: show the dilution of samples from 10^1 -10^9, Lane 4: molecular mass DNA marker (100bp) and Lane 11: negative control for the 3 pathogens.

Interestingly, simultaneous specific detection of all pathogens of all target strains including *Salmonella* Typhimurium (ATCC® 14028™), *Bacillus cereus* (ATCC® 10876™) and S*taphylococcus aureus* (ATCC® 25923™) could be successfully achieved down to 10^1 CFU/ml (Fig. 1), and the same as in *Listeria monocytogenes* (NCINB® 50007), *E. coli* (ATCC® 25922) and *Campylobacter* (ATCC® 29428) (Fig.2). The multiplex assay developed in this study was effective for the detection of target pathogens in both broth and spiked meat.

Multiplex PCR protocol detection of artificially contaminated samples

To validate the multiplex PCR assay for its application to food samples, the minimum enrichment incubation time and limit of detection

of the multiplex PCR using peptone medium were conducted. Meat product inoculated with *Salmonella* Typhimurium (ATCC® 14028™), *Bacillus cereus* (ATCC® 10876™), *Staphylococcus aureus* (ATCC® 25923™), *Listeria monocytogenes* (NCINB® 50007), *E. coli* (ATCC® 25922) and *Campylobacter* (ATCC® 29428) with nine levels of the number of viable cells (from10^9 to 10^1 CFU/ml) were analyzed as well as a non-inoculated sample as negative control. The results demonstrated that after 24-h enrichment (Fig. 3 and 4), the multiplex PCR assay was able to correctly identify the presence of the three food-borne pathogens at all different inoculated levels and down to the lowest concentration of 10^1 CFU/ml in all meat samples.

DISCUSSION

Food borne pathogens from family *Enterobacteriaceae* are most common pathogens which are found in most raw and processed food products and this demand apply assays for investigation. Recently PCR assays reported to be the rapid, sensitive and low cost method in comparison with tradition method of isolation.

We used to detect six different food born bacteria in two multiplex reactions (*Salmonella, S. aureus, Bacillus cereus*, *Listeria monocytogenes, E. coli* and *Campylobacter*) with critical chosen step of primer pair order to ensure specificity and sensitivity and to avoid cross-reactions. Taking into consideration that they should have similar melting temperatures (Tm) and also selected very important target genes.

Many mPCR assays are done for simultaneous detection of different pathogens, a mPCR assays have been developed for the detection of *Bacillus cereus, Staphylococcus aureus, Listeria monocytogenes, E. coli* O157:H7 and *Salmonella* spp. (Thapa et al., 2012), also a mPCR assays for detection of *Salmonella* spp*., Escherichia coli* O157, and *Listeria monocytogenes*(Suo et al., 2010) beside simultaneous determination of DNA from *Escherichia coli*, *Clostridium perfringens, Staphylococcus aureus* and *Bacillus cereus* in food samples (Koppel et al., 2017) and detection of *Escherichia coli* O157:H7, *Staphylococcus aureus* and *Salmonella* (Wei et al., 2018).

Selection of pathogen-specific target genes and the design of compatible PCR primers are critical steps to achieving high detection, specificity and avoidance of the competition in the multiplex target amplifications (Suo et al., 2010). In this assay we selected a great important in virulence of these bacteria. *InvA* is a virulence gene encoding an invasion protein and a common unique gene in all strains of *salmonella* spp. (Rahn et al., 1992). *clfA* gene for Binding of *S. aureus* to fibrinogen (Fg) which encode cell-associated protein clumping factors (*ClfA* and *B*) (McDevitt et al., 1994; Eidhin et al., 1998). Heat shock in *Bacillus subtilis* induce as many as 66 proteins after temperature up shift from 37 ° to 48°C,identified as the homologues for *GroEL, DnaK*, enolase, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH)(Miller et al., 1991). 16S rRNA has been targeted due to a single base pair difference in the V9 region of the sequence of the genes coding for ribosomal RNA which distinguished *L. monocytogenes* from other closely related *Listeria* spp*. (Czajka* et al., 1993*;* Kumar et al., 2015*).* Alkaline phosphatase (AP) is a periplasmic protein comprised of 50kDa subunits and is encoded by the *phoA* gene of *E. coli* (Bradshaw et al., 1981; Chang et al., 1986)and *23s rRNA* contains more information because of its greater length (De Rijk et al., 1992)) contain a maximum of variable helices. Indeed, when investigating evolutionary relationships between very closely related organisms, highly variable regions provide most of the information(Van Camp et al., 1993)) but Park et al., (2006)used specific *Salmonella* spp. specific primer *Its* gene, S. aureus specific primer *Cap8A-B*. Also Cheng et al., (2012) used for *Staphylococcus aureus, Salmonella enterica, Bacillus cereus* and *Vibrio parahaemolyticus* specific primer *SAOUHSC_02297, inv*A, *hbl* and *tlh*A genes respectively.

The practical application of these two multiplex PCR assay was tested using spiked meat samples containing pathogens with different dilution of pathogens, it's very sensitive and not affected by the presence of other macromolecules such as fat, protein, and carbohydrates in the reaction system that might have obstructed the amplification reaction of the target sequences. The results indicate the limits for detection of *Salmonella, S. aureus* and *Bacillus subtilis* together *and Listeria monocytogenes, E. coli* and *Campylobacter together* in the meat was 1 CFU per one g food sample after enrichment for 24 hours. And there is excellent agreement between results of the multiplex PCR and the conventional culture method obtained in agree with Babu et al., (2013) that detected 10 CFU of each of *Salmonella* spp., *Escherichia coli* and *Shigella* spp. inoculated in 1 g of a homogenized food

sample in 50 ml BHI broth and lee et al., (2014) who detected *Escherichia coli* O157:H7*, Bacillus cereus, Vibrio parahaemolyticus, Salmonella* spp*., Listeria monocytogenes,* and *Staphylococcus aureus* as few as 1 CFU /ml of each pathogen after enrichment for 12 h. While Wei et al., (2018) detected *E. coli* O157:H7, *S. aureus*, *Salmonella* as 10^3 CFU/ml in the culture broth, whereas it was 10^4 CFU/ml in the model food system. Also Kawasaki et al., (2009) detected *Salmonella* spp*., Listeria monocytogenes,* and *Escherichia coli* O157:H7 from inoculated sample after 20 hours of enrichment ≤5 CFU/25 g.

CONCLUSION

Since food borne pathogens detection techniques are very important nowadays for food industry as traditional detection methods depend on several steps bacterial culture in combination with biochemical tests, a process that takes 4 to 7 days to complete for each bacteria. We established a study to help the issue of time in traditional methods by developing a multiplex PCR assay for each of three food borne pathogenic bacteria within 24h. This current study consists of a simultaneous detection of *Salmonella, S. aureus, Bacillus cereus, Listeria monocytogenes*, *E*. *coli* and *Campylobacter* in a single reaction, with high accuracy and efficiency beside careful choice of PCR primers to increase sensitivity.

CONFLICT OF INTEREST

The present study was performed in absence of any conflict of interest.

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AUTHOR CONTRIBUTIONS

ZAE: collected the samples, did the bacterial isolation and identification and wrote the paper.
AME: mPCR.
SAN, RE and JE: group supervisors.
All authors read and approved the final version.

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