Simultaneous detection of seven foodborne Enterobacteriaceae pathogens using multiplex PCR

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ABSTRACT

Members of the family Enterobacteriaceae are major pathogens associated with gastrointestinal disorders caused by the consumption of contaminated foods. This study developed a multiplex PCR targeting specific genes for simultaneous detection and differentiation of seven major Enterobacteriaceae members, namely, *Escherichia coli* (*uid*A), *Proteus sp.* (*atp*D), *Salmonella* sp. (*inv*A), *Enterobacter sp.* (16S rRNA), *Klebsiella sp.* (*gyr*A), *Klebsiella pneumoniae* (*rpo*B) and *K. oxytoca* (*peh*X) from contaminated food samples. Four main types of food samples comprise fresh, frozen, processed frozen and processed preserved samples were examined by using simultaneous amplification of those seven-targeted genes from obtained reference strains. The multiplex PCR assay showed detection sensitivity reached 5-10 cfu for each pathogen after 2h of sample incubation on nutrient broth. Generally, the 7plex-PCR assay revealed that the most common pathogens found in 192 different food samples with high frequency was *Salmonella* which distributed within most of all types of food having the maximum occurrence frequency reached 6.77% in hotdog and sausage samples followed by 5.21% in fresh chicken and beef burger. The occurrence frequency % of *E. coli* was found to be high in chicken/beef burger samples (6.25%), and then slightly decreased to 5.21%, 4.68% and 4.17% in frozen meat, hotdog/sausage and fresh fish samples, respectively. The processed preserved (chicken/beef luncheon and pastrami) and frozen chicken possessed the lowest presence of foodborne pathogens. This study recommends the use of 7plex-PCR detection system for the rapid and accurate detection of some Enterobacteriaceae pathogens is inexpensively and thus could be used for the regular monitoring of food quality.

Key words: Multiplex PCR, Enterobacteriaceae, Foodborne pathogens.

INTRODUCTION

Foodborne disease (FBD) outbreaks are common and often cause considerable morbidity and mortality. Foodborne illnesses are infections or irritations of the gastrointestinal tract caused by food or beverages that contain harmful bacteria, viruses, molds, worms and protozoa. The two most common types of food borne diseases are intoxication and infection. Intoxication occurs when toxin produced by the pathogens cause food poisoning, while infection is caused by the ingestion of food containing pathogens (Grant *et al*., 2008). When the toxin ingested along with the food, it gives rise to the Food Poisoning Syndrome (Havelaar *et al*., 2015). Transmission and sources of foodborne diseases are from plant surfaces, animals, water, sewage, air, soil or from food handlers during handling and processing (Bean *et al*., 1990).

Enterobacteriaceae foodborne pathogens as *Salmonella* species, *Proteus* species and some serotypes of *Escherichia coli* continue to be the major pathogens associated with food poisoning outbreaks. Also, *Klebsiella pneumoniae* is most commonly associated with

nosocomial infections and most frequently causes pneumonia, urinary tract, central nervous system, hepatic infections, wound and/or blood infections (Podschun *et al*., 1998) and associated with intestinal infections, with symptoms of diarrhea if enterotoxigenic strains are involved (Klipstein and Engert 1976). *Salmonella* species causes enteric fever in humans and fatal diseases, gastroenteritis and other extra-intestinal complications (D'Aoust, 1989). *Enterobacter* species are widely distributed in nature found in the soil, water, dairy products, and in the intestines of animals as well as humans (Euzéby *et al*., 1997).

With the continuing impact of these pathogens on human and animal health, the need for rapid and accurate detection methods for these organisms both in environmental and clinical samples remains high. Moreover, identification of enterobacteriaceae members including *E. coli*, *Salmonella sp.*, *Proteus sp.*, *Enterobacter sp.* and *Klebsiella sp.*, is important for surveillance, prevention and control of food-borne diseases. An accurate and rapid procedure for identification would help identify the sources, reservoirs and or their transfer through food chain.

Conventional methods for pathogen detection based on culturing, selective enrichment and biochemical testing are generally cumbersome, inefficient, labor intensive and time consuming take 3-5 days or more (Miescier *et al.*, 1992; D'Aoust, 1989). In contrast, advanced DNA-based methods, such as DNA hybridization and PCR-based assays (mono and multiplex PCR, real-time PCRs), fluorescent DNA probes, microarrays and DNA fingerprinting techniques, , ELISA-based assays are rapid and highly sensitive. Those techniques could be applied universally for identification of food-borne pathogens because they rely on the nucleic acid composition of the bacterium instead of their phenotypic expressions that may be variable under culture conditions (Olsen *et al*., 1995; Batt, 1997).

The PCR represents a rapid procedure with both high sensitivity and specificity for the immediate detection, identification of specific pathogenic bacteria from various food matrices as individual detection (monoplex PCR) (Lantz *et al*., 1994; Hill *et al*., 1996) and multiplex PCR (Way et al., 1993; Soumet et al., 1999). The invasion antigen gene (*invA*) was used for detection of *Salmonella sp.* (Rahn *et al*.,1992; Malkawi and Gharaibeh 2003; Trafny *et al*., 2006; Litty *et* al. 2013). The multiplex PCR had been used successfully for detection of *Salmonella enterica* and differentiate all four *Shigella* spp using *invA* gene and *ipaH1* gene, respectively (Radhika *et al.*, 2014). The ß-glucuronidase (*uidA*) was specific for *E. coli* detection (Li *et al.* 2005; Anklam *et al*. 2012; Litty *et al*. 2013). A certain and specific primers derived from *gyrA* gene was used for detection of *Klebsiella* spp (Yogesh et al. 2011; Brisse and Verhoef, 2001). Furthermore, one can differentiate between *K. pneumonia* and *K. oxytoca* using certain primers for amplifying *rpoB* and *pehX* target genes, respectively (Kovtunovych et al. 2003; Yogesh et al. 2011). *Proteus* species were detected using a target segment of ATP synthase subunit beta (*atpD*) gene Shui-lianBi *et al*. 2013). The simultaneous multiplex PCR detection for five different food borne pathogens (Jeong *et al*., 2007; Zheng *et al*., 2013; Litty *et al*., 2013), and six food borne pathogens (Beili *et al*., 2013) were performed successfully. Unfortunately, the detection of more than six organisms in the same reaction by using multiplex PCR is quite obstacle. This study tried to optimize a standard condition for 7plex-PCR in order to rapid detect seven target food born Enterobacteriaceae pathogens.

MATERIALS AND METHODS

Food samples, bacterial isolation and culturing

Four main types of food samples were collected from different stores and markets at Damietta County, Egypt. The food samples which suspected having contamination with Enterobacteriaceae pathogens comprised 40 fresh food samples (chicken, beef meat and fish), 50

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frozen food samples (chicken, beef meat and filet of-fish), 60 processed frozen food samples (hotdog/sausage and chicken/beef burger), and 42 processed preserved food samples (chicken/beef luncheon and pastrami).

Presumptive Enterobacteriaceae colonies were selected and isolated on three chromogenic differential media including Salmonella-Shigella (SS), Eosin Methylene Blue (EMB) (Levine's formulation), and Triple Sugar Iron (TSI) media. 1gm of food sample was added to 5mL of sterile nutrient broth media then whirly mixed and incubated for 2h at 30°C for enrichment. 200mL of each serial dilution of each sample were speeded on the previously mentioned media. After overnight incubation, the resulting colonies were examined, isolated and purified as a single colony for further identification. Another 200mL was centrifuged and kept in -20°C freezer for chromosomal DNA extraction and PCR analysis.

The isolated bacterial strains were identified morphologically, physiologically and biochemically according to Bergey`s Manual of Systematic Bacteriology (Bernner and Farmer 1984; Bercovier and Mollaret 1984; Patrick and Francine 1984; Rowe and Gross 1984).

Standard bacterial strains

The reference bacterial strains (*E. coli*, *Proteus sp.*, *Salmonella sp.*, *Enterobacter sp.*, *Klebsiella sp.*, *Klebsiella pneumonia* and *Klebsiella oxytoca*) were obtained from the culture collection of Microbiology Laboratory, Faculty of Science, Damietta University.

DNA Extraction

Chromosomal DNA was extracted by phenol/chloroform technique according to procedure modified from (Ausubel *et al.*, 1996). 1.5mL of bacterial cell suspension was centrifuged at 6.500rpm for 5min. The pellet was suspended in 1ml SET buffer (20% sucrose, 50mM EDTA, 50mM Tris-HCl, pH 7.6). The pellet was re-suspended in 100μL buffer (SET buffer + Lysozyme), vortex and followed by the addition of 2μL RNase. After incubation at 37°C for 10min, 500μL TE buffer (10mM Tris-HCl, pH 7.6, 0.2mM EDTA) and 70μL of 10% sodium dodecyl sulfate were added. DNA was extracted with 500μL of phenol/ chloroform/isoamyl alcohol solution (25:24:1), centrifuged at 6.500rpm for 5min and precipitated with isopropanol. The DNA was harvested by centrifugation at 6.500rpm for 10min, washed with 500μL of 75% cold ethanol and the DNA pellet was suspended in 1/10 TE buffer and stored at -20°C until used for PCR amplification.

Primers and PCR conditions

The oligonucleotide sequence of the primers and the expected sizes for each pathogen are represented in Table 1. For optimization of the multiplex PCR amplification, 30 *p*mole of each primer, 2μL of extracted chromosomal DNA and 25μL 2x Mytaq Red Mix (BIOLINE) were used. The final volume of the reaction mixture was adjusted to 50μL with water free nuclease. The multiplex PCR reactions were performed in the thermal cycler (TECHNE TC-312, UK) with initial denaturation at 95°C for 5 minutes followed by 35 cycles of denaturation at 95°C for 30 seconds, primer annealing at 58°C for 1 minute, primer extension at 72°C for 1:30 minute, and the final extension at 72°C for 10 minutes.

Table 1. List of primers and the expected amplicon sizes for each target gene and pathogen.

Primer	Sequence (5'-3')	Amplicon size (bp)	Target gene	Bacterial strain	Reference
UidA-F **UidA-R**	For: GTCACGCCGTATGTTATTG Rev: CCAAAGCCAGTAAAGTAGAAC	530	*uidA*	*E. coli*	Litty, et al. (2013)
AtpD-F **AtpD-R**	For:GTATCATGAACGTTCTGGGTAC Rev: TGAAGTGATACGCTCTTGCAG	595	*atpD*	*Proteus sp.*	Shui-lianBi, et al. (2013)
InvA-F **InvA-R**	For: GTGAAATTATCGCCACGTTCGGGCAA Rev: TCATCGCACCGTCAAAGGAACC	284	*invA*	*Salmonella sp.*	Rahn, et al. (1992)
16s DNA-F **16s DNA-R**	For: ATGTCTGGGAAACTGCCTGATG Rev: CGGGTAACGTCAATAGACAAGG	372	16S rRNA	*Enterobacter sp.*	Tajbakhsh, et al. (2015)
GyrA-F **GyrA-R**	For: CGCGTACTATACGCCATGAACGTA Rev: ACCGTTGATCACTTCGGTCAGG	441	*gyrA*	*Klebsiella sp.*	Brisse and Verhoef (2001)
RpoB-F **RpoB-R**	For: CAACGGTGTGGTTACTGACG Rev: TCTACGAAGTGGCCGTTTTC	108	*rpoB*	*Klebsiella pneumonia*	Yogesh, et al. (2011)
PehX-F **PehX-R**	For: GATACGGAGTATGCCTTTACGGTG Rev: TAGCCTTTATCAAGCGGATACTGG	343	*pehX*	*Klebsiella oxytoca*	Kovtunovych, et al. (2003)

Agarose gel electrophoresis and DNA detection

The detection of genomic DNA and size of PCR products were examined by agarose gel electrophoresis. 3% agarose was dissolved in TAE buffer pH 8.0 (0.04M Tris-acetate and 0.001M EDTA). 2μL of ethidium bromide (10 mg/mL) was added for DNA staining. Usually 2μL of loading buffer (0.25% Bromophenol blue, 70% glycerol, 10 mM Tris-HCl pH 7.0) was added to the sample. 50bp DNA Ladder was used as molecular marker. The DNA on the gel was visualized by UV transilluminator.

Sensitivity of multiplex PCR primers

The previous sets of primers were subjected to study their sensitivity towards the minimal cell count detection. In order to perform that, 1ml serial dilutions (10^0 to 10^{-5}) of the reference bacterial strains were set from their overnight cultures on nutrient broth medium. 100μl aliquots of each dilution (in triplicates) was cultured on nutrient agar plates for colony forming unit (cfu) count. Other 100μl aliquots of the same dilutions was centrifuged for bacterial cell collection. The DNA from each pellet was extracted and then subjected to PCR analysis. The resulting PCR products were compared with the corresponding cfu count for each dilution.

RESULTS

Identification and distribution of the foodborne pathogens

A total of 192 food samples comprised 40 fresh food samples (chicken, beef meat and fish), 50 frozen food samples (chicken, beef meat and filet-o-fish), 60 processed frozen food samples (hotdog/sausage and chicken/beef burger), and 42 processed preserved food samples (chicken/beef luncheon and pastrami). The classical identification based on morphological, physiological and biochemical characteristics, in addition to their distribution within the different types of food samples were listed in Table (2) and Figure (1). Generally, *Salmonella* is the most dominant genus, which distributed within most of all types of food having the maximum occurrence frequency reached 6.77% in hotdog and sausage samples followed by chicken and

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beef burger with 5.21%. The frequency of *E. coli* with found to be high in fresh fish samples (4.17%), and then slightly decline to 3.13% in frozen chicken and meat samples.

The multiplex PCR

The seven sets of oligonucleotide primers were examined separately using reference bacterial strains as mentioned in the material and methods section. The amplified DNA fragments of the expected molecular for each strain was examined by agarose gel electrophoresis as shown in Figure 2. The individual PCR products were represented in lane 1-7, while the multiplex PCR for the seven tested sets of primers was in lane 8.

Sensitivity of the optimized multiplex PCR

The sensitivity of the multiplex PCR assay was determined (Fig. 3). The results of multiplex PCR assay were able to detect the cultivable pathogens up to 5-10 cfu bacterial count which found in the 10^{-4} culture dilution of each reference bacterial strains. The culture dilution 10^{-5} containing less than 5 cfu could not detect any amplification for the targeted DNA.
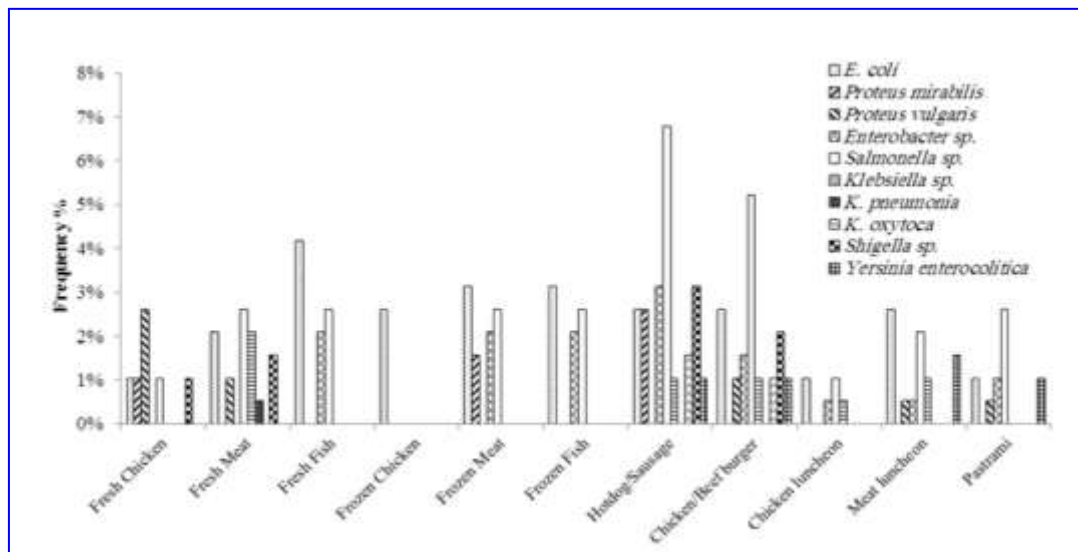

Fig. 1. The occurrence frequency and distribution of the identified bacterial pathogens found in the different types of food samples.

Table 2. The occurrence frequency and distribution of the identified bacterial pathogens found in the different types of food sample.

Type of samples		Fresh			Frozen			Processed frozen		Processed preserved		
		Chicken	Meat	Fish	Chicken	Meat	Fish	Hotdog/Sauage	Chicken/ Beef burger	Chicken luncheon	Meat luncheon	Pastrami
No. of samples		11	13	16	11	20	19	30	30	12	15	15
No. of negative samples		2	4	3	6	5	6	5	9	8	6	7
Pathogen's frequency %	E. coli	1.04%	2.1%	4.17%	2.6%	3.13%	3.13%	2.6%	2.6%	1.04%	2.6%	1.04%
	Proteus mirabilis	1.04%	0%	0%	0%	1.56%	0%	2.6%	0%	0%	0%	0%
	Proteus vulgaris	2.6%	1.04%	0%	0%	0%	0%	0%	1.04%	0%	0.52%	0.52%
	Enterobacter sp.	0%	0%	2.1%	0%	2.1%	2.1%	3.13%	1.56%	0.52%	0.52%	1.04%
	Salmonella sp.	1.04%	2.6%	2.6%	0%	2.6%	2.6%	6.77%	5.21%	1.04%	2.1%	2.6%
	Klebsiella sp.	0%	2.1%	0%	0%	0%	0%	1.04%	1.04%	0.52%	1.04%	0%
	K. pneumonis	0%	0.52%	0%	0%	0%	0%	0%	0%	0%	0%	0%
	K. oxytoca	0%	0%	0%	0%	0%	0%	1.56%	1.04%	0%	0%	0%
	Shigella sp.	1.04%	1.56%	0%	0%	0%	0%	3.13%	2.1%	0%	0%	0%
	Yersinia enterocolitica	0%	0%	0%	0%	0%	0%	1.04%	1.04%	0%	1.56%	1.04%
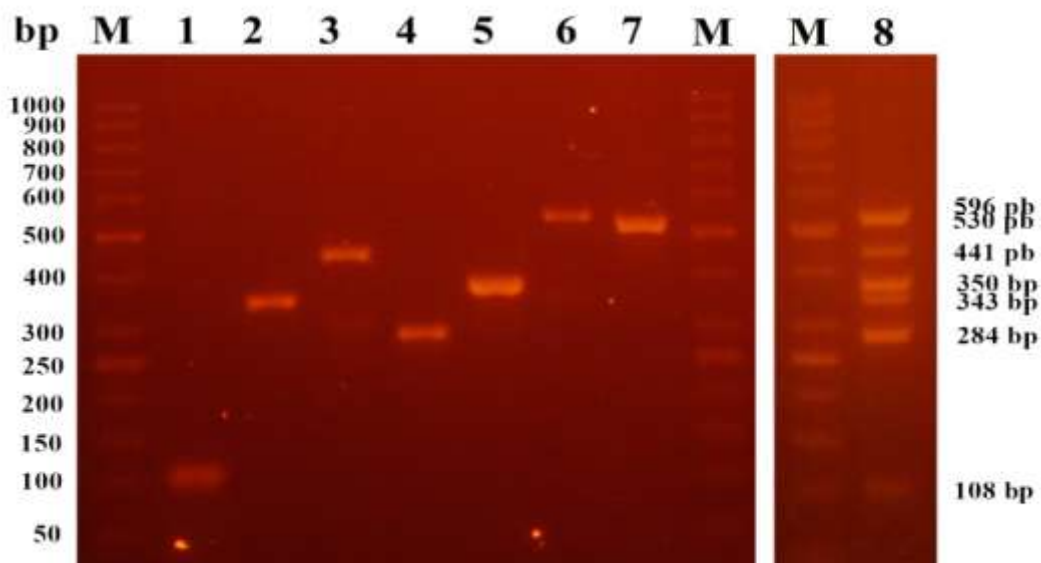
Fig. 2. Agarose gel electrophoresis for the individual PCR amplification of the seven target gene from purified DNA of the standard seven microbial pathogens (lane 1, *Klebsiella Pneumonia*; lane 2, *Klebsiella oxytoca*; lane 3, *Klebsiella Sp.*; lane 4, *Salmonella sp.*; lane 5, *Enterobacter sp.*; lane 6, *Proteus sp.*; lane 7, *E. coli*) and the multiplex PCR for the all seven gene targets (lane 8). Lane M is the 50 bp DNA marker.

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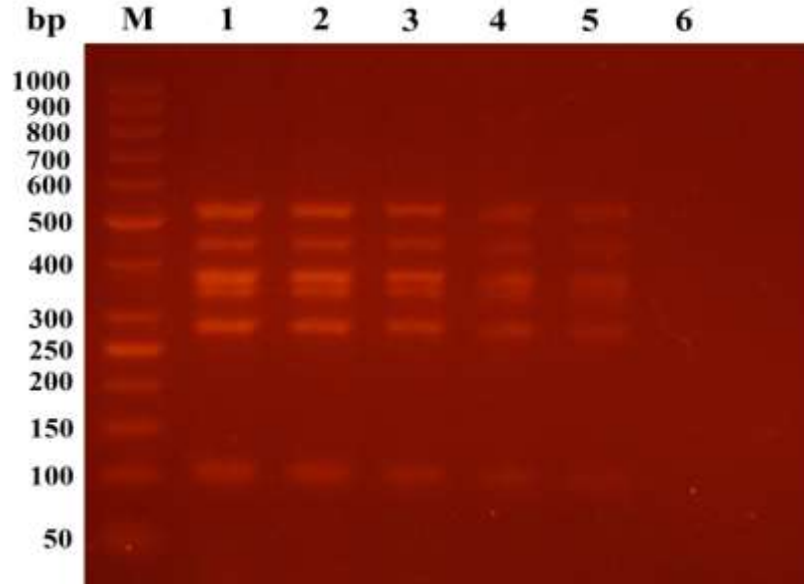

Fig. 3. Sensitivity of the multiplex PCR applied to seven target genes of the standard pathogens. Lanes 1 to 6, bacterial dilutions, 10^0 - 10^{-5}, respectively. Lane M, 50 bp DNA marker.

7plex-PCR detection for fresh food pathogens

The multiplex PCR assay for 40 fresh food samples (chicken, beef meat and fish) is represented in Table 3 and Figure 4 as the sizes of the amplified target DNA for each tested pathogen gave the expected and correct size. Three fresh meat samples were highly contaminated with three pathogens (*E. coli*, *Salmonella sp.* and *Klebsiella sp.*). The species name of *Klebsiella* in those three samples did not recognize as neither *pneumonia* nor *oxytoca*. Both of *E. coli*, and *Salmonella sp.* were detected in 4 and 5 fresh chicken and meat samples, respectively. The occurrence frequency % (Fig. 5) of *Salmonella sp.* was the highest (25%) in the meat samples followed by *E. coli* (20%) in the fish samples. The presence of *K. pneumonia* was the lowest (2.5%) within meat samples, while *K. oxytoca* could not be detected within any fresh food samples.

Table 3. The different multiplex PCR patterns for 40 fresh samples of chicken, beef meat and fish. (+: positive PCR results, ND: not detected samples).

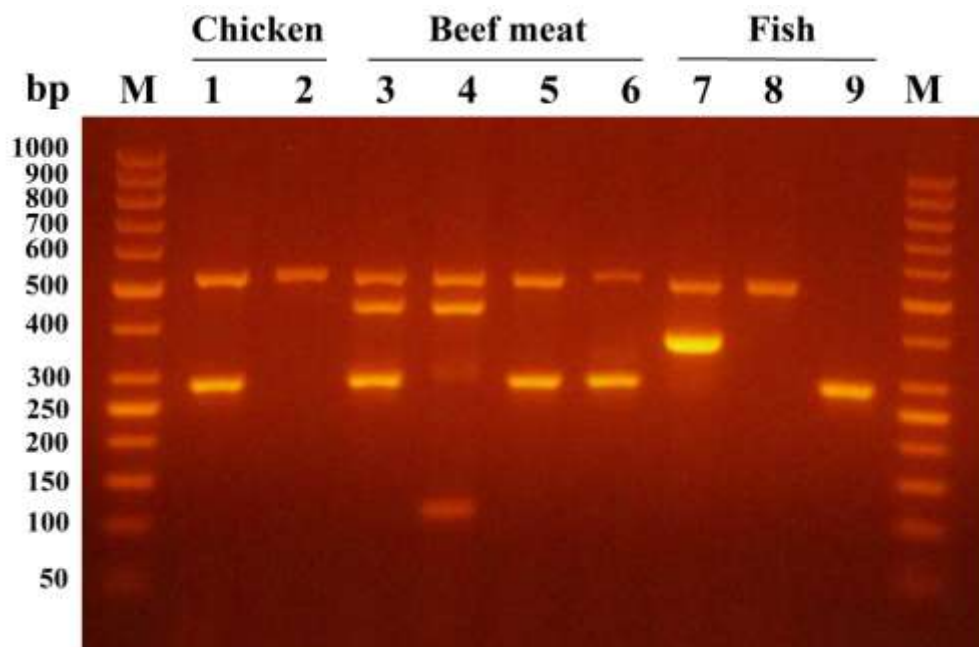| Type of samples	Chicken			Beef meat					Fish			
PCR pattern	1	2	ND	3	4	5	6	ND	7	8	9	ND
No. of samples	4	5	2	3	1	3	4	2	4	4	5	3
E. coli	+			+	+	+			+	+		
Proteus sp.		+					+					
Enterobacter sp.									+			
Salmonella sp.	+			+		+	+				+	
Klebsiella sp.				+	+							
Klebsiella pneumonia					+							

Fig. 4. Agarose gel electrophoresis pattern for the multiplex PCR of contaminated fresh food samples (chicken, beef meat and fish). Lane1, *E. coli* **and** *Salmonella sp.***; lane 2,** *Proteus sp.***; lane 3,** *E. coli, Klebsiella. sp.* **and** *Salmonella sp.***; lane 4,** *E. coli, Klebsiella. sp.***,** *K. pneumonia***; lane 5,** *E. coli* **and** *Salmonella sp.***; lane 6,** *Proteus sp.* **and** *Salmonella sp.***; lane 7,** *E. coli* **and** *Enterobacter sp.***; lane 8,** *E. coli***; lane 9***, Salmonella sp*.
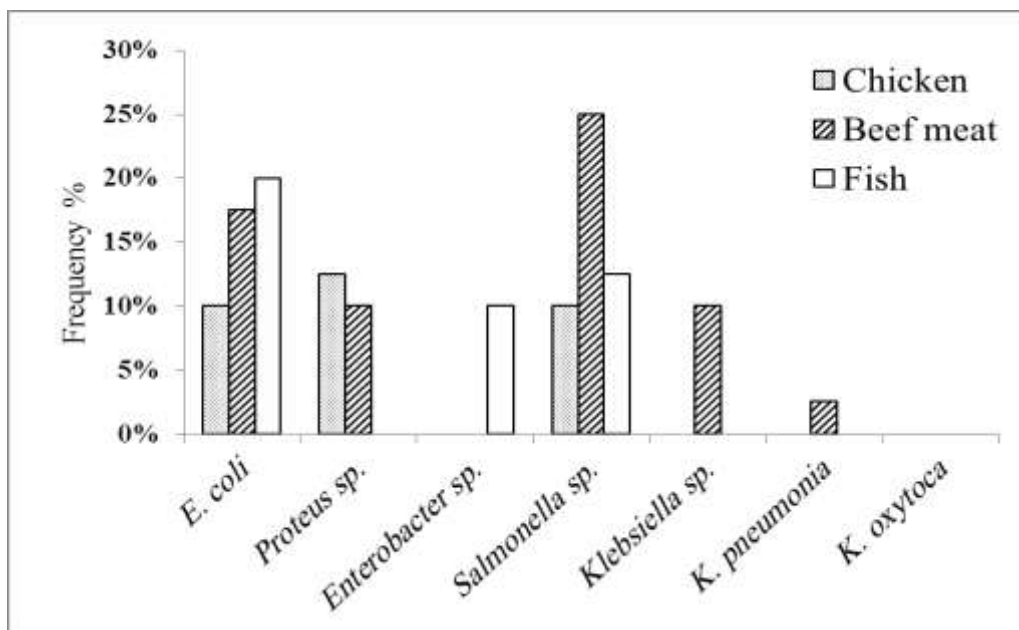
Fig. 5. Frequency percentage of the occurrence for the bacterial pathogens found in 40 fresh food samples (chicken, beef meat and fish) as detected by the multiplex PCR technique.

7plex-PCR detection for frozen food pathogens

A total of 50 frozen food samples (chicken, beef meat and filet of fish) were examined by multiplex PCR assay where their patterns are represented in Table 4 and Figure 6. Out of 11 frozen chicken samples, only 5 were contaminated with *E. coli*. Three of meat and two filets of fish frozen samples were contaminated with both of *E. coli*, and *Salmonella sp.* as detected by the multiplex PCR. The *Enterobacter sp.* was found in 4 filets of fish samples and associated with *E. coli* in 4 beef meat samples. The occurrence frequency % (Fig. 7) of *E. coli.* was the highest (20% and 12.5%) in the beef meat and filets of fish frozen samples, then decline to 10% in the chicken samples. In addition, *Salmonella sp.* was found in beef meat and filet of fish with frequency reached 10%. The presence of *Proteus sp.* was the lowest (6%) within the beef meat samples. The multiplex PCR assay could not detect any species of *Klebsiella* within the frozen food samples.

Table 4. The different multiplex PCR patterns for 50 frozen samples of chicken, beef meat and filet-o-fish. (+: positive PCR results, ND: not detected samples).

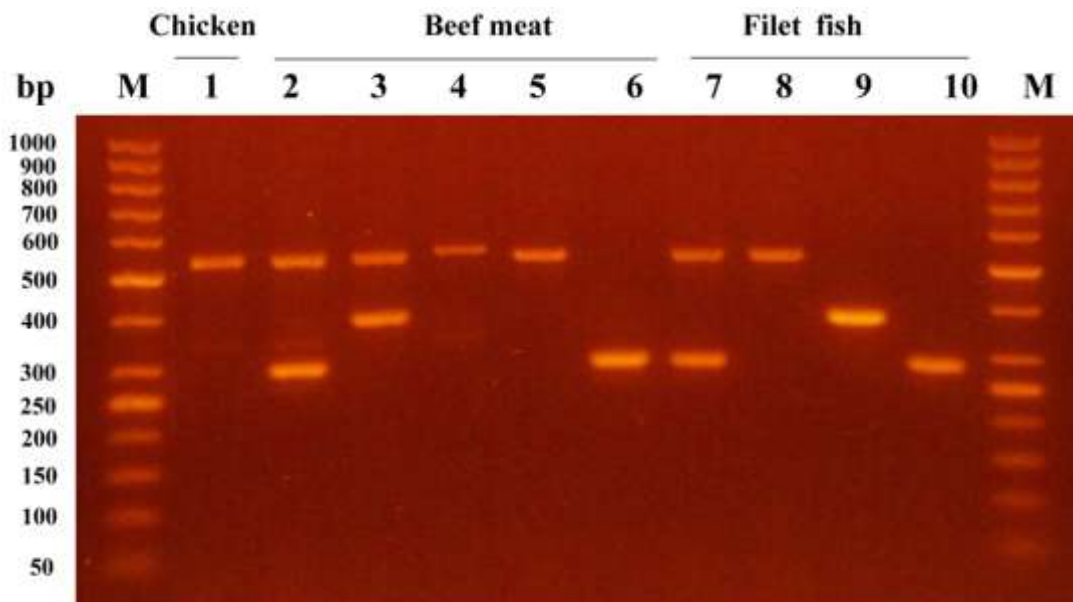| Type of samples	Chicken		Beef meat						Filet-o-fish				
PCR pattern	1	ND	2	3	4	5	6	ND	7	8	9	10	ND
No. of samples	5	6	3	4	3	3	2	5	2	4	4	3	6
E. coli	+		+	+		+			+	+			
Proteus sp.					+								
Enterobacter sp.				+							+		
Salmonella sp.			+				+		+			+	

Fig. 6. Agarose gel electrophoresis pattern for the multiplex PCR of contaminated frozen food samples (chicken, beef meat and filet-o-fish). Lane1, *E. coli*; lane 2, *E. coli* and *Salmonella sp.*; lane 3, *E. coli* and *Enterobacter sp.*; lane 4, *Proteus sp.;* lane 5, *E. coli*; lane 6, *Salmonella sp.*; lane 7, *E. coli* and *Salmonella sp.*; lane 8, *E. coli*; lane 9, *Enterobacter sp.*; lane 10, *Salmonella sp.*
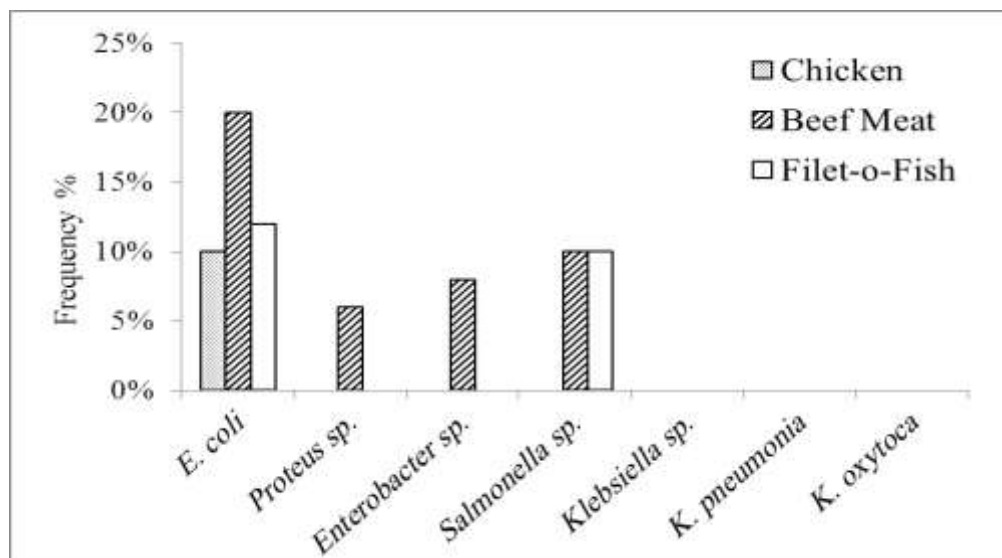
Fig. 7. Frequency percentage of the occurrence for the bacterial pathogens found in 50 frozen food samples (chicken, beef meat and filet-o-fish) as detected by the multiplex PCR technique.

7plex-PCR detection for processed frozen food pathogens

Sixty processed frozen food samples of hotdog/sausage and beef/chicken burger were examined by multiplex PCR assay. Their different patterns are represented in Table 5 and Figure 8. The highest contamination with more than individual pathogens were detected in 4 hotdog/sausage samples as they contain three bacterial pathogens (*E. coli, Enterobacter sp*. and *Salmonella sp*.), also, 2 beef/chicken burger samples possessed three pathogens included *E. coli, Salmonella sp*. and *Klebsiella oxytoca*). The occurrence frequency % (Figure 9) of *Salmonella sp*. was the highest (21.7% and 20%) followed by *E. coli* (15% and 20%) found in hotdog/sausage and beef/chicken burger samples, respectively. The multiplex PCR could not detect *K. pneumonia* within the processed frozen food samples.

Table 5. **The different multiplex PCR patterns for 60 processed frozen samples of hotdog/sausage and beef/chicken burger. (+: positive PCR results, ND: not detected samples).**

Type of samples	Hotdog and sausage							Beef and chicken burger						
PCR pattern	1	2	3	4	5	6	ND	7	8	9	10	11	12	ND
No. of samples	4	3	2	3	5	6	5	2	3	3	2	7	4	9
E. coli	+	+	+					+		+		+		
Proteus sp.						+					+			
Enterobacter sp.	+		+						+					
Salmonella sp.	+	+				+		+	+	+			+	
Klebsiella sp.				+				+						
Klebsiella oxytoca				+				+						

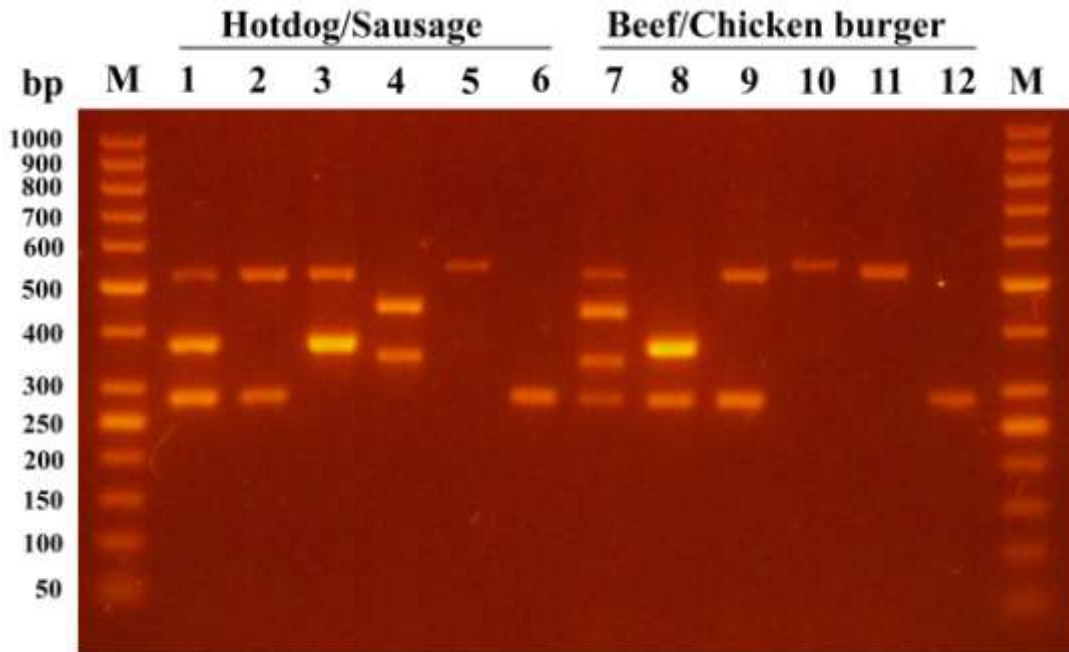Simultaneous detection of seven foodborne Enterobacteriaceae pathogens using multiplex PCR

Fig. 8. Agarose gel electrophoresis pattern for the multiplex PCR of contaminated processed food samples (hotdog/sausage and beef/chicken burger). Lane1, *E. coli,* *Enterobacter sp.* **and** *Salmonella sp.***; lane 2,** *E. coli* **and** *Salmonella sp.***; lane 3,** *E. coli* **and** *Enterobacter sp.***; lane 4,** *Klebsiella sp.* **and** *Klebsiella oxytoca;* **lane 5,** *Proteus sp.***; lane 6,** *Salmonella sp.***; lane 7,** *E. coli, Salmonella sp., Klebsiella sp.* **and** *Klebsiella oxytoca***; lane 8,** *Enterobacter sp.* **and** *Salmonella sp.***; lane 9,** *E. coli* **and** *Salmonella sp.***; lane 10,** *Proteus sp.;* **lane 11,** *E. coli***; lane 12,** *Salmonella sp***.**
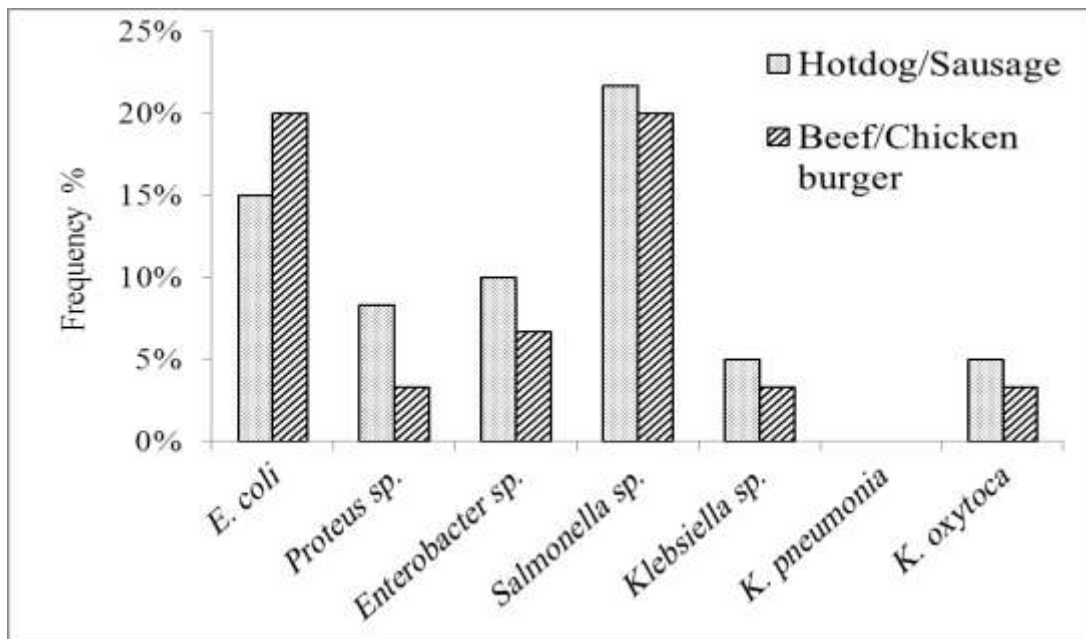
Fig. 9. Frequency percentage of the occurrence for the bacterial pathogens found in 60 processed food samples (hotdog/sausage and beef/chicken burger) as detected by the multiplex PCR technique.

7plex-PCR detection for processed preserved food pathogens

The different multiplex PCR patterns for 42 processed preserved food samples (chicken luncheon, meat luncheon and pastrami) are represented in Table (6) and Figure (10). Two meat luncheon samples were highly contaminated with three different pathogens comprise *E. coli, Salmonella sp.* and *Klebsiella sp.*, while another two samples of meat luncheon and pastrami were contaminated with *E. coli,* and *Salmonella sp.* One samples of chicken luncheon contained *E. coli,* and *Enterobacter sp.* The other samples were either contaminated with individual pathogen or not contaminated as assayed by the multiplex PCR technique. The frequency % of pathogens presence within the processed preserved food samples in Figure 11 showed that the *E. coli* possessed the highest frequency (16.7%) in meat luncheon followed by *Salmonella sp.* (11.9%) in pastrami samples. The multiplex PCR could not detect *K. pneumonia* and *K. oxytoca* within the processed preserved food samples.

Table 6. The different multiplex PCR patterns for 42 processed preserved samples of chicken luncheon, meat luncheon and pastrami. (+: positive PCR results, ND: not detected samples).

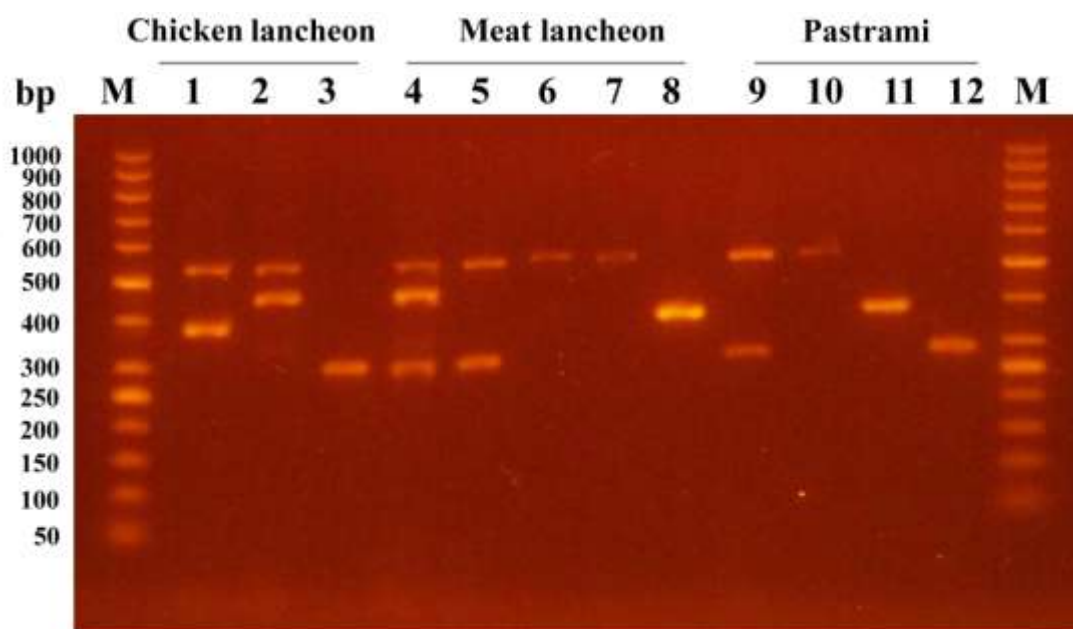| Type of samples	Chicken luncheon				Meat luncheon						Pastrami				
PCR pattern	1	2	3	ND	4	5	6	7	8	ND	9	10	11	12	ND
No of samples	1	1	2	8	2	2	1	3	1	6	2	1	2	3	7
E. coli	+	+			+	+		+			+				
Proteus sp.							+					+			
Enterobacter sp.	+								+				+		
Salmonella sp.			+		+	+					+			+	
Klebsiella sp.		+			+										

Fig. 10. Agarose gel electrophoresis pattern for the multiplex PCR of contaminated preserved processed food samples (chicken luncheon, meat luncheon and pastrami). Lane1, *E. coli* **and** *Enterobacter sp.***; lane 2,** *E. coli*

DISCUSSION

Despite the improvements in food processing hygiene practices in recent years, the occurrence of foodborne pathogenic microorganisms is still commonplace. Certain members of the family Enterobacteriaceae are predominant pathogens that found in raw and processed food products and this presence demands prompt investigation (Anbazhagan *et al.*, 2010). Therefore, appropriate methods could be optimized and applied to routine monitoring for pathogens in food and food-processing environments to improve disease control and reduce risks to human health (Moon *et al*., 2004). The conventional methods of isolation and identification of these pathogens primarily depends on selective media followed by a battery of biochemical tests that are laborious, costly and time consuming (Radji *et al*., 2010). The multiplex PCR strategy has the potential of being relatively low-cost assays with the additional advantages being reliable and rapid (Hübner *et al.,* 1992; Varshney *et al.,* 2007; González-Escalona *et al*. 2009; Chen *et al.* 2012). This study concerned to perform a rapid and simultaneous detection of seven foodborne pathogens for four different food categories including fresh food (chicken, beef meat and fish), frozen food (chicken, beef meat and filet-o-fish), processed frozen food (hotdog/sausage and chicken/beef burger), and processed preserved food (chicken/beef luncheon and pastrami) using 7-plex PCR assay.

The multiplex PCR using seven sets of primer was able to detect the total of 5-10 cfu of each pathogen. Tantawiwat *et al*., (2005) were able to detect 1 to 10 cfu for *E. coli*, *Clostridium perfringens* and *Klebsiella pneumonia* using multiplex PCR after pre-enrichment step by culturing for 6 hr. otherwise the sensitivity was rather low to 10^4 cfu/ ml. Maheux *et al.,* (2011) were able to detect 4-5 enterococcal colony forming units using real time PCR. According to Litty *et al*. (2013), multiplex PCR detection limit was 10 cfu per reaction after 10 h of incubation. Ojha et al. (2013) demonstrated that the sensitivity of pentaplex PCR was 120 cfu per reaction mixture of bacteria for detection of different *Shigella* species. Furthermore, Zheng et al., (2014) reported that simultaneous detection of five major foodborne pathogens in pork was developed by designing five pairs of primers *invA* gene, *hlyA* gene, *rfbE* gene, *nuc* gene and *ail* gene for detecting *Salmonella*, *Listeria. monocytogenes*, *E. coli O157:H7*, *Staphylococcus aureus* and *Yersinia. enterocolitica*, respectively. After overnight culture, the detection limit was less than 10 cfu/mL for detection of a single pathogen and 103 cfu/mL for the simultaneous detection of the five target species. Detection of very low levels of bacterial contamination in food necessitates that these samples to be cultured for a few hours in nutrient broth for providing conditions for growth and multiplication of bacterial pathogens to a detectable level. Furthermore, dilution of inhibitory substances present in food and dilution of dead target cells, which provides some assurance that the detected DNA belongs from viable target cell. In this study, 1 gm of the food sample was diluted in 10 mL nutrient broth and incubated for 2 h for enticement.

Comparison of bacterial occurrence frequencies in the food samples between the use of cultural traditional and advanced multiplex techniques revealed some little differences. This might be attributed to the none viability of some bacterial strains that could not able to be grown, isolated and identified classically giving positive detection results by m-PCR rather than cultural methods. In contrast, the positive detection of culture methods that gave negative when m-PCR was applied might be due to the low concentration of bacterial pathogens which could not be detected by m-PCR.

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The result revealed that the highest frequency percentage of the detected pathogens occurred in the processed frozen food samples (hotdog/sausages and chicken/meat burger) followed by fresh food samples. This probably due to many reasons including the low level of hygiene during the handling procedures during the transportation and/or food processing, the inadequate biosafety storage and shelf life. Therefore, the highest frequency percentage of the bacterial occurrence was recorded for *Salmonella* species and *E. coli*, which are the most common foodborne pathogens.

This study recommend the use of 7plex-PCR detection system for the rapid, accurate and unambiguous identification and detection of *E. coli, Proteus sp., Enterobacter sp., Salmonella sp., Klebsiella sp., K. pneumoniae* and *K. oxytoca* foodborne pathogens. Furthermore, this m-PCR assay could be used in hospitals and clinical laboratories for routine detection purposes once the method is fully evaluated on a large number of blood/fecal samples.

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الكشف المتزامن لسبعة من الإنتيروبكتيريا الممرضة المصاحبة للأطعمة باستخدام تفاعل البلمرة المتسلسل المتعدد

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المستخلص

تعتبر أفراد عائلة البكتيريا المعوية أحد أهم مسببات الأمراض الرئيسية المرتبطة بالاضطرابات المعدية المعوية الناتجة عن استهلاك الأطعمة الملوثة. فى تلك الدراسة تم إستخدام تقنية تفاعل البلمرة المتعدد باستهداف جينات مخصصة للكشف المتزامن فى آن واحد لسبع من أعضاء البكتيريا المعوية المصاحبة لتلوث الأغذية المسماة كالتالى:

Escherichia coli (*uid*A), *Proteus sp.* (*atp*D), *Salmonella* sp. (*inv*A), *Enterobacter sp.* (16S rRNA), *Klebsiella sp.* (*gyr*A), *Klebsiella pneumoniae* (*rpo*B) and *K. oxytoca* (*peh*X).

تم فحص أربعة أنواع رئيسية من عينات الطعام و التى اشتملت على عينات الطازجة ومجمدة ومصنعة مجمدة ومصنعة محفوظة باستخدام الإكثار المتزامن لتلك الجينات السبعة المستهدفة من السلالات المرجعية التي تم الحصول عليه. أظهر اختبار PCR المتعدد حساسية الكشف وصلت cfu 10-5 لكل مسببات الأمراض بعد ساعتين من تحضين العينة على مرق مغذى. بشكل عام أظهر فحص PCR أن أكثر مسببات الأمراض شيوعًا في 192 عينة مختلفة من الأغذية كان السالمونيلا *Salmonella* التى كانت منتشرة داخل معظم أنواع جميع الأغذية والتي بلغ نسبة تواجدها 6.77٪ في عينات الهوت دوج والسجق متبوعة بنسبة 5.21٪ في الدجاج الطازج ولحم البقر برجر، أما نسبة تواجد بكتيريا الإشيريشيا كولى *Escherichia coli* فكان في عينات برجر الدجاج/اللحم البقري بنسبة 6.25٪ ، ثم انخفض بشكل طفيف إلى 5.21٪ ، 4.68٪ و 4.17٪ في اللحوم المجمدة، عينات الهوت دوج /النقانق والأسماك الطازجة، على التوالي. توصي هذه الدراسة باستخدام نظام الكشف PCR المتعدد و المتزامن للكشف السريع والدقيق عن بعض مسببات الأمراض المعوية Enterobacteriaceae ، وبالتالي يمكن استخدامها للمراقبة المنتظمة لجودة الأغذية.