Detection of *Salmonella typhimurium* in Different Food Sources by Conventional Method and multiplex PCR assay

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Abstract

The occurrence of foodborne diseases is increasing throughout the world. Bacteria of the genus Salmonella are responsible for food poisoning and, in some cases, may be fatal. The aim of this study was to adapt the multiplex PCR technique (mPCR) on the rapid and direct identification of the presence of *Salmonella* sp. and *Typhimurium* in different food sources. *Salmonella* detection by both conventional culture and mPCR methods were performed on 400 samples collected over a 7-month period between December 2013 and June 2014 from street vendors, exposed foods that are sold on the sidewalks, and in popular restaurants. *Salmonella* isolates were identified by a multiplex-PCR using three sets of primers targeting the *inv*A , *Mdh* and *fliC* genes sequences from *Salmonella* spp. and *S. typhimurium* respectively. The results of culture method indicated that 73 samples (18.25%) out of the 400 showed positive results, and displayed that (10)40% of the examined frozen meat, (9)36% of minced meat, (16) 64% of frozen chicken, (5)20% of hamburger, (6)24% of fresh kebab, (4)16% of salad and ice cream, (3)12% of each basturma, fruit Cocktail, orange juice and raisin juice, (2)8% of mayonnaise and tabbouleh were contaminated with *Salmonella* Spp., whilst pomegranate juice and watermelon were not contaminated. The traditional method for the detection of *Salmonella* reveals *Salmonella* and bacteria-like *Salmonella*, a Serological detection was used to distinguish the *Salmonella* only. The results indicate 61 samples (83.56 %) out of the 73 were *Salmonella* spp., and 13(30.14%) samples out of 61 were *Salmonella typhimurium.* The results of mPCR indicated that 61 samples (15.25%) out of the 400 demonstrated positive results for the invA target gene as *Salmonella* spp. The results displayed that (8)32% of the examined frozen meat, (13)52% of frozen chicken, (6)24% of minced meat and fresh kebab, (4)16% of hamburger and salad, (3)12% of each basturma, Chickpea, fruit cocktail and raisin juice (2) 8% of each Mayonnaise, Tabbouleh, orange juice and ice cream were contaminated with *Salmonella* Spp., whilst pomegranate juice and watermelon not contaminated. On the other hand 22 samples (5.5%) out of the 400 demonstrated positive results for Mdh target gene as *salmonella typhimurium*. The results displayed that(5) 20% of the examined frozen meat, (7)28% of frozen chicken, (4)16% of minced meat (2)8% of each hamburger and fresh kebab, and (1)4% of basturma and salad were contaminated with

Salmonella typhimurium, whilst other plant products, beverage and ice cream were not contaminated with *Salmonella typhimurium.* Multiplex PCR successfully amplified the DNA fragments corresponding in size as fallows *Salmonella* spp.389 bp (*invA* target gene), *Salmonella typhimurium* 261bp (*Mdh* target gene) , but not from all the non-Salmonella. *fliC* gene did not work when used with *invA* and *Mdh* genes*,* may be due to its large size and need different temperature cycles. Our conclusion was that the traditional method is laborious , time consuming and less accurate because it detects *Salmonella* and bacteria-like *Salmonella*. Whilst mPCR was found to be a very sensitive test that allowed rapid and reliable identification of these bacteria in food and beverage samples. Results of this study can be used by agriculture and health organizations in Iraq.

Keywords: *Salmonella* Spp., *Salmonella typhimurium*, Food, Beverage, Culture method, mPCR.

Introduction

Various agents including bacteria, viruses and parasites cause foodborne diseases. About 38.6 million illnesses are caused by known pathogens every year of which 36% are due to foodborne organisms . Bacterial infections account for approximately thirty percent of the total foodborne illnesses of which, non- typhoidal *Salmonella* infections contribute to the highest percent of the mortality. Approximately 95% of human *Salmonella* infections are food borne with *S. typhimurium* bacteria being acquired from contaminated food (Mead *et al*., 1999). *Typhimurium* is the most frequently isolated serovar worldwide (Madadgar *et al*., 2008). Dairy products, vegetables, fruits, shellfish, beef, poultry and eggs are the most common sources of human salmonellosis ((Molbak, 2005; Doyle and Beuchat, 2007). Consumption of food contaminated by these pathogens has not only caused numerous infections but also resulted in numerous foodborne outbreaks(Suo *et al.,* 2010). The overall incidence of laboratory confirmed *Salmonella* Healthy People 2010 objective of 6.8 cases per 100,000 persons (Matyas *et al*.,2010). According to EFSA in 2009 4:4% of food-borne illnesses were reported which were associated with fruit, vegetables, their juices, and their products (Hausdorf, 2012) .*Salmonella* spp. are typically found in soil, water, food, and the gastrointestinal tract of humans and other animals (Anderson and Ziprin, 2001).

All *Salmonella* strains possess an invasion gene (*invA*), which encodes proteins for adherence and invasion. Therefore, *Salmonella* can penetrate the gut lumen into the epithelium cells of host small intestine (Galan *et al.*, 1992). The *fliC* gene (a flagellar gene of *Salmonella)* is responsible for the expression of a flagellar protein specific to *S. typhimurium* (Soumet *et al*., 1999).

The identification of the genus *Salmonella* using conventional bacteriological analyses is laborious and time consuming and not compatible with routine

processing of large numbers of samples (Mainali *et al*., 2011; Kataria *et al*., 2013). based on cultures using selective media and characterization of suspicious colonies by biochemical and serological tests (Kumar ,2012). Moreover, morphological changes and the biochemical profile of the colonies both increase the uncertainty of this diagnosis (Fach *et al*.,1999). Serovar confirmations using traditional methodologies must be performed in reference laboratories and in a country as large as Iraq only the Central Public Health Laboratory (CPHL) in Baghdad can perform these analyses, causing significant research delays. Consequently, the introduction of methodologies that could securely identify the principal serovars of *Salmonella* would be very impor-tant for rapid diagnoses . Therefore, a rapid methods is necessary for identification of *Salmonella* serotypes from foods. mPCR is one such methods. Multiplex PCR was found to be a very sensitive test that allowed rapid and reliable identification of *salmonella* bacteria (das Chagas *et al*., 2013). de Freitas *et al*. (2010) reported that the results of *Salmonella* sp. detection in refrigerated viscera showed that the mPCR was able to detect *Salmonella* genus in 2.74% of 200 samples. The multiplex PCR (mPCR) is a variation of reaction chain polymerase (Kumar *et al*., 2008), which involves more than one pair of primers allowing one single reaction to detect more than one type of microorganism along with their serotypes and/or different genes, therefore reducing the diagnosis period (Malkawi and Gharaibeh, 2003). PCR technology represents a rapid procedure with high sensitivity and high specificity to detect *Salmonella* in a wide variety of food. Multiple microorganisms can be detected with a multiplex-PCR (mPCR) which can amplify several target genes in one PCR run (Kawasaki *et al*., 2009). Several PCR assays have been developed by targeting various Salmonella genes, such as *invA* (Burkhalter *et al*., 1995 ; Akbarmehr , 2011), *fliC* gene (Akbarmehr , 2011**;** das Chagas *et al*., 2013). **)***iroB* (Baumler *et al*., 1997), 16S rRNA , agfA , and viaB and virulence-associated plasmids [Loongyai *et al*., 2010 ; Kataria *et al*., 2013], genes encoding for rRNA (Iida *et al*., 1993) or *Salmonella*-septic DNA sequences with an unknown function (Aabo *et al*., 1993).

Poor sanitation of school's street foods (that are obtained from street vendors outside schools), exposed foods that are sold on the sidewalks, and in popular restaurants which is commonly found in Iraq, may lead to the disease that could risk human health. Therefore, *Salmonella typhimurium* is an important food hygiene indicator to access the quality of street foods. In recent years several studies were carried out by different authors in order to determine the prevalence of salmonellosis in Iraq. But until now epidemiological study about salmonellosis in baghdad province - Iraq has not been widely studied. Therefore in the present study we investigated the salmonellosis in different food sources in baghdad province using conventional culture and mPCR assay.

Material and Methods

Collection of samples

Through the period extending from December 2013 till June 2014, A total of 400 different food and beverage samples were collected, 25 sample of each (Frozen meat, Minced meat, Frozen Chicken, Hamburger, Basturma, Fresh Kebab, Salad, Chickpea, Mayonnaise, Tabbouleh, Fruit Cocktail, Pomegranate juice, Melon juice, Orange juice, Raisin juice, and ice Cream) from street vendors, exposed foods that are sold on the sidewalks, and in popular restaurants, Baghdad, Iraq. Samples were collected using sterile bags and transported to the Central Public Health Laboratory (CPHL) in Baghdad for detection of pathogenic bacteria (*Salmonella ser. Typhimurium*).

Preparation of Samples

Allot of 400 food and beverage samples were collected from street vendors, exposed foods that are sold on the sidewalks, and in popular restaurants, Baghdad, Iraq. samples were selected for the possibility of contamination of *Salmonella* during the handling, processing and storage of raw material of the foods and beverages. All samples that were labeled and recorded have to be analyzed as soon as possible. Samples can be refrigerated on 0-4 °C for not more than 24 h after collection. The pre-enrichment of samples was performed according to (ISO, 2002). Briefly, twenty five g of cheese sample was placed in 225 ml of nutrient Broth for the enrichment, incubated for 24 hours at 37° C.

Transfer one ml from the mix into Tetrathionate broth and Selenite Sistein broth, incubated for 24 hours at 37°C. Loop full of the enrichment broth was cultured on selective media (XLD), incubated for 24 hours at 37°C. The pre-enrichment culture was then divided into two aliquots. The first aliquot was subjected to DNA extraction by boiling method and the second aliquot was used to confirm the presence of *Salmonella* by standard cultural method, and followed by biochemical and serological confirmatory tests.

DNA Extraction

A volume of 1.5 ml of the post-enriched sample was centrifuged at 14,000g for 1 min, DNA was extracted using Presto Mini g DNA Bacteria Kit according to manufacturer's instructions(Geneaid, Korea). The extracted DNA was stored −20 ◦C until use.

Agarose Gel

After genomic DNA extraction, agarose gel electrophoresis was adopted to confirm the presence and integrity of the extracted DNA. Ten µl portion of the sample was analyzed by electrophoresis in agarose gel (2%), staining with ethidium bromide (Promega, USA), and visualized in UV light. A DNA molecular weight standard 50 bp was analyzed along with the samples (Wang *et al.,* 1997).

Specific primers

The primers listed in table 1 were selected for this study; these primers were provided in a lyophilized form, dissolved in sterile distilled water to give a final

concentration of 100pmoles/ µL as recommended by provider and stored in deep freezer until used in PCR amplification.

Table 1. The sequences and conventional of forward and reverse primer

Species	Target gene	PCR primers' sequences (5' – 3')	Product size
Salmonella spp.	*invA gene*	invA F(5-GCTGCGCGCGAACGGCGAAG-3)	389-bp
		invA R (5-TCCCGGCAGAGTTCCCATT-3)	
Salmonella Typhimurium	*Mdh gene*	mdh F: 5′ TGCCAACGGAAGTTGAAGTG	261bp
		mdh R: 5′ CGCATTCCACCACGCCCTTC	
Salmonella Typhimurium	*fliC gene*	fliC F 5′ CGG TGT TGC CCA GGT TGG TAA T	620bp
		fliC R 5′ ACT GGT AAA GAT GGC T	

MPCR assay :DNA amplification and detection

Purified DNA was used as a templet for the PCR assay. For the m- PCR, three primer pairs were used. The polymerase chain reaction (PCR) amplification was performed in a final volume of 20µl of reaction mixture containing 12.5 µL of PCR Master Mix, 1.0 µL of each of the primers and 50 ng of genomic DNA , 1.5 mM MgCl2 and 1U of *Taq* DNA polymerase. transported the samples to a thermal cycle using the amplification program consisting of initial denaturation at 94°C for 5 min, 1 cycles with a denaturation at 94°C for 1min , 35 cycle , each of which consisted of holding at 58°C, for 1 min, 60°C for 1 min, 62°C for 1 min, 64°C for 1 min, 66°C for 1 min and 68°C for 1 min for primer annealing and extension, at 72°C for 2 min , followed by the final extension at 72°C for 10 min , 1 cycle. The multiplex PCR products were added to the loading buffer (Bioneer) and submitted to electrophoresis in 1.5% agarose gel (Promega). The gel was prepared using TBE buffer colored with ethidium bromide (10 mg/ml) (Promega). The products amplified sizes were identified using 100 base pair DNA ladder (Bioneer). The amplified products were visualized under ultra violet light.

Statistical Analysis

The Statistical Analysis System- SAS (2012) was used for the evaluation of the effect of different factors in study parameters. Chi-square test was used to compare between the percentage in this study at 1% and 5% probability level.

Results and Discussion

Results

Detection by Traditional Method

The results indicate that 73 samples (18.25%) out of the 400 were positive results (Table 2). All kinds of food, beverage and ice cream were contaminated with *Salmonella* in varying degrees with the exception of pomegranate juice and watermelon, which were not contaminated. Frozen chicken, frozen meat, and minced meat were most polluted with *Salmonella* and differ significantly (**χ2** =13.56) from plant products. In general, meat products were the more contaminated than plant products (Table 2).

The microbiological procedure used for the detection of studied bacteria in food, beverage and ice cream were performed according to protocols of *Salmonella* organism. The results of culture method displayed that 64% of the examined frozen chicken, 40% of frozen meat, 36% of minced meat, 20% of hamburger, 24% of fresh kebab, 16% of salad and ice cream, 12% of each basturma, fruit Cocktail, orange juice and raisin juice, 8% of mayonnaise and tabbouleh were contaminated with *Salmonella* Spp., whilst pomegranate juice and watermelon not contaminated (Figure 1).

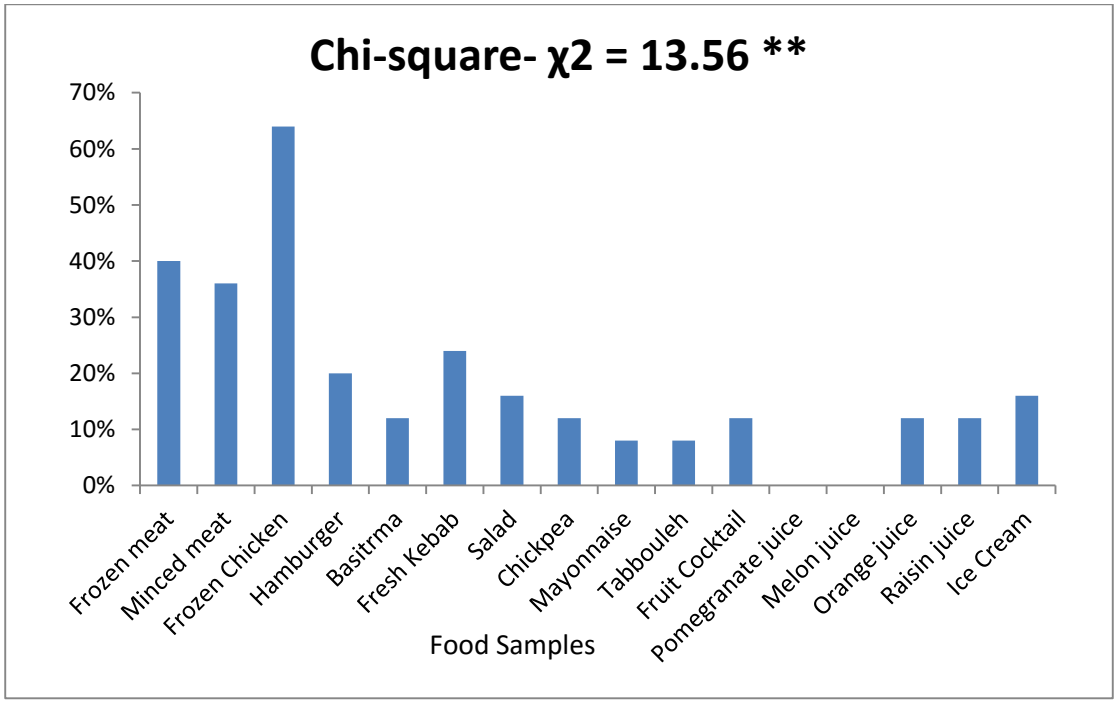

Figure 1. Percentage of *Salmonella* spp isolated from food samples by using the traditional method.

Depending on morphology, round pale colony with black center on XLD agar (Fig. 2), and the outcome of biochemical test clarified that the 3 isolates of *Salmonella* Spp., fermented glucose not lactose, appeared as red surface and yellow bottom of KIA with gas and H2O formation.

The traditional method for the detection of *Salmonella* reveal *Salmonella* and bacteria-like *Salmonella*. A Serological detection was used to distinguish the *Salmonella* only. Serological identification of *Salmonella* spp. established the presence of *Salmonella* spp. in food,

beverage and ice cream samples. The results indicate sixty one samples (83.56 %) out of the 73 were *Salmonella* spp., and 13 samples out of 61 were *Salmonella typhimurium* (Table 3). Serological examination showed that the highest contamination of food with bacteria was by *salmonella typhimurium* (30.14%) followed by *salmonella anatum* (20.55%) (Table. 3).

Table 2. *Salmonella* spp isolated from food samples by using the traditional method.

N. of sample / Type of food	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	Total	%
Frozen meat	-	+	+	+	+	-	-	+	+	+	-	-	-	+	+	+	-	-	-	-	-	-	-	-	-	10	40
Minced meat	-	+	-	+	+	+	-	+	-	+	-	+	-	+	-	-	-	-	-	-	+	-	-	-	-	9	36
Frozen Chicken	+	+	-	+	-	+	+	+	+	+	+	-	-	-	-	+	-	+	+	+	-	-	+	+	+	16	64
Hamburger	-	+	-	-	+	-	-	-	+	-	-	-	-	-	+	-	-	-	+	-	-	-	-	-	-	5	20
Basturma	-	-	-	-	-	-	-	+	-	+	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	3	12
Fresh Kebab	-	+	-	-	+	+	-	-	-	+	-	-	-	-	-	+	+	-	-	-	-	-	-	-	-	6	24
Salad	-	-	-	-	-	+	-	+	-	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	4	16
Chickpea	-	-	-	-	-	-	-	-	+	+	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	3	12
Mayonnaise	+	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	2	8
Tabbouleh	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	2	8
Fruit Cocktail	-	-	-	-	+	-	-	-	-	-	+	-	-	-	-	-	-	+	-	-	-	-	-	-	-	3	12
Pomegranate juice	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0	0
Melon juice	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0	0
Orange juice	-	-	-	-	+	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	+	-	-	-	-	3	12
Raisin juice	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+	-	-	-	-	-	-	-	-	3	12
Ice Cream	-	-	-	+	-	+	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	+	4	16
Total																										73	18.25
Chi-square- χ^2	-------																									---	13.56 **

** (P<0.01).

Figure 2. The shape of *Salmonella* in food sample

Table 3.Serological identification of Salmonella serotype

No.	Species	No.	Species
1	*salmonella enteritidis*	45	***salmonella Typhimurium***
2	*salmonella anatum*	46	***salmonella Typhimurium***
3	*salmonella enteritidis*	47	***salmonella Typhimurium***
4	*salmonella Dublin*	48	***salmonella Typhimurium***
5	*salmonella Dublin*	49	***salmonella Typhimurium***
6	*salmonella anatum*	50	***salmonella Typhimurium***
7	*salmonella anatum*	51	***salmonella Typhimurium***
8	*salmonella anatum*	52	***salmonella Typhimurium***
9	*salmonella anatum*	53	***Citrobacter spp***
10	*salmonella anatum*	54	***Proteus spp***
11	*Proteu sspp*	55	***Citrobacter spp***
12	*Citrobacter spp*	56	***Citrobacter spp***
13	*salmonella Typhimurium*	57	***Citrobacter spp***
14	*salmonella Typhimurium*	58	***Citrobacter spp***
15	*salmonella Typhimurium*	59	***salmonella ohio***
16	*salmonella Typhimurium*	60	***Salomnella enteritidis***
17	*salmonella Typhimurium*	61	***salmonella anatum***
18	*salmonella Dublin*	62	***salmonella anatum***
19	*salmonella Typhimurium*	63	***salmonella Typhimurium***
20	*salmonella Typhimurium*	64	***salmonella ohio***
21	*salmonella Typhimurium*	65	***salmonella braenderup***
22	*salmonella Typhimurium*	66	***salmonella braenderup***
23	*salmonella Newport*	67	***salmonella braenderup***
24	*salmonella Newport*	68	***salmonella braenderup***

25	*salmonella enteritidis*	69	***salmonella braenderup***
26	*salmonella enteritidis*	70	***salmonella braenderup***
27	*salmonella hato*	71	***salmonella anatum***
28	*salmonella hato*	72	***salmonella anatum***
29	*salmonella Typhimurium*	73	***salmonella braenderup***
30	*salmonella Typhimurium*		
31	*proteusspp*		
32	*proteusspp*		
33	*salmonella Typhimurium*		
34	*salmonella Typhimurium*		
35	*salmonella hato*		
36	*salmonella hato*		
37	*proteusspp*		
38	*proteusspp*		
39	*Salomnella ohio*		
40	*salmonella anatum*		
41	*salmonella anatum*		
42	*salmonella anatum*		
43	*salmonella anatum*		
44	*salmonella anatum*		

Detection by Molecular Method

DNA Extraction from Food, Beverage and ice cream samples

The total DNA from each sample was successfully obtained from the pre-enrichment broth and using simple protocol genomic DNA kit. A high yield of purified DNA can be isolated, the DNA quality and integrity were estimated through remaking the DNA bands by electrophoresis on agaros 1% for 30 min. The bands appear sharp single not diffused and have no smear which may result from DNA degradation as shown in figure 4.9. The total DNA was used as templates in mPCR reactions for *Salmonella* spp., and serotype *Typhimurium* research.

DNA Purity and Concentration

DNA concentration and purity were assessed by the use of nanodrop. The observed quantities of total DNA ranged between 5-8ng/µl and purity ranging from 1.7-2 which indicates the presence of pure DNA.

Detection by m PCR

The first step in adapting PCR protocol for gene amplification is to choose the DNA extraction procedure to get a target DNA which is suitable for applying in PCR experiment. The results of this study indicated that DNA extraction kit procedure for food, beverage and ice cream samples is giving pure DNA.

As indicated in table 4, a variety of food products were selected and procured for

the detection of *Salmonella Spp*. using mPCR technique. The results indicate sixty one samples (15.25%) out of the 400 demonstrated positive results for the *invA* target gene as *Salmonella* spp. (Table 4). All kinds of food and beverage were contaminated with *Salmonella* spp. in varying degrees with the exception of pomegranate juice and watermelon, which were not contaminated .Frozen chicken, frozen meat, minced meat and fresh kebab were most polluted with *Salmonella* and differ significantly **(P<0.01)** from plant products (χ^2 =11.07) . In general , meat products were the more contaminated from plant products(Table 4). The results of displayed that 32% of the examined frozen meat, 52% of frozen chicken, 24% of minced meat and fresh kebab, 16% of hamburger and salad ,12% of each basturma, Chickpea, fruit cocktail and raisin juice 8% of each Mayonnaise , Tabbouleh , orange juice and ice cream were contaminated with *Salmonella* Spp. , whilst pomegranate juice and watermelon not contaminated (Figure 3).

On the other hands twenty two samples (5.5%) out of the 400 demonstrated positive results for *Mdh* target gene as *salmonella typhimurium* **(**Table 4).The results indicated that frozen chicken, frozen meat , meat products and salad were contaminated with *Salmonella typhimurium*. Whereas other plant products, beverage and ice cream were not contaminated with *Salmonella typhimurium*. The results of displayed that 20% of the examined frozen meat, 28% of frozen chicken, 16% of minced meat 8% of each hamburger and fresh , and 4% of Basturma and salad were contaminated with *Salmonella typhimurium*. , whilst other plant products, beverage and ice cream were not contaminated with *Salmonella typhimurium* (Figure 3).

In this study the multiplex PCR assays employ three sets of primers for the genes one targeting *invA* , one targeting *fliC* and one targeting *Mdh* . Multiplex PCR successfully amplified the DNA fragments corresponding in size as fallows *Salmonella* spp.389 bp **(***invA* target gene**),** *Salmonella typhimurium* 261bp **(** *Mdh* target gene) (figure 4) but not from all the non-Salmonella. *fliC* gene did not work when used with *invA* and *Mdh* genes*,* may be due to its large size and need different temperature cycles.

Table 4. *Salmonella typhimurium* isolated from food samples by using mPCR method

N. of sample / Type of food	Gene	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	Total	%
Frozen meat	**invA**	-	-	+	+	-	-	-	+	+	+	-	-	-	+	+	+	-	-	-	-	-	-	-	-	-	**8**	**32**
	Mdh	-	-	-	+	-	-	-	+	-	+	-	-	-	-	+	+	-	-	-	-	-	-	-	-	-	**5**	**20**
Minced meat	**invA**	-	+	-	+	+	-	-	-	-	+	-	-	-	+	-	-	-	-	-	-	+	-	-	-	-	**6**	**24**
	Mdh	-	-	-	+	+	-	-	-	-	+	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	**4**	**16**
Frozen Chicken	**invA**	+	+	-	-	-	-	+	+	+	+	-	-	-	-	-	+	-	+	+	+	-	-	+	+	+	**13**	**52**
	Mdh	-	-	-	-	-	-	+	-	-	+	-	-	-	-	-	+	-	+	-	-	-	-	+	+	+	**7**	**28**
Hamburger	**invA**	-	+	-	-	+	-	-	-	-	-	-	-	-	-	+	-	-	-	+	-	-	-	-	-	-	**4**	**16**
	Mdh	-	-	-	-	+	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	**2**	**8**
Basitrma	**invA**	-	-	-	-	-	-	-	+	-	+	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	**3**	**12**
	Mdh	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	**1**	**4**
Fresh Kebab	**invA**	-	+	-	-	+	+	-	-	-	+	-	-	-	-	-	+	+	-	-	-	-	-	-	-	-	**6**	**24**
	Mdh	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	**2**	**8**
Salad	**invA**	-	-	-	-	-	+	-	+	-	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	**4**	**16**
	Mdh	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	**1**	**4**
Chickpea	**invA**	-	-	-	-	-	-	-	-	+	+	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	**3**	**12**
	Mdh	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	**0**	**0**
Mayonnaise	**invA**	+	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	2	**8**
	Mdh	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	**0**	**0**
Tabbouleh	**invA**	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	**2**	**8**
	Mdh	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	**0**	**0**
Fruit Cocktail	**invA**	-	-	-	-	+	-	-	-	-	-	+	-	-	-	-	-	-	+	-	-	-	-	-	-	-	**3**	**12**
	Mdh	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	**0**	**0**
Pomegranate juice	**invA**	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	**0**	**0**
	Mdh	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	**0**	**0**
Melon juice	**invA**	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	**0**	**0**

	Mdh	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	**0**	**0**
Orange juice	**invA**	-	-	-	-	+	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	**2**	**8**
	Mdh	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	**0**	**0**
Raisin juice	**invA**	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+	-	-	-	-	-	-	-	**3**	**12**
	Mdh	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	**0**	**0**
Ice Cream	**invA**	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	**2**	**8**
	Mdh	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	**0**	**0**
Total																										**83**	**10.37**
Chi-square- χ^2		-------																								---	**11.07****
Total	**invA**																									**61**	**15.25%**
	Mdh																									**22**	**5.5%**
Chi-square- χ^2																											**8.92 ****
** (P<0.01).																												

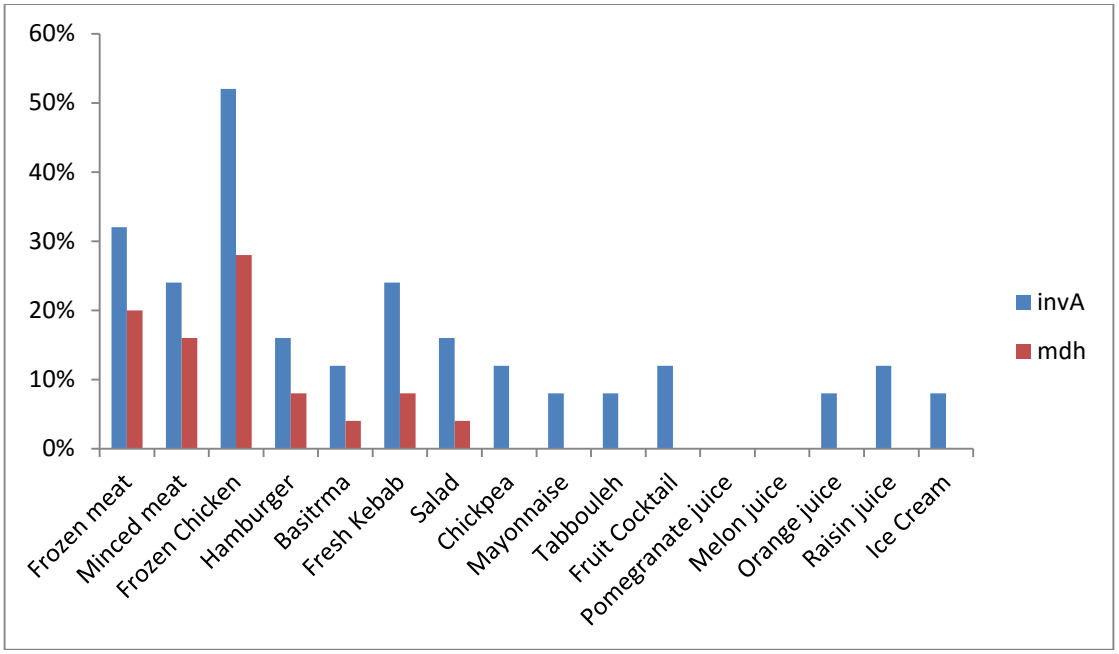

Figure 3. The percentages of *Salmonella* spp. and *Salmonella typhimurium* detected in food, beverage and ice cream.

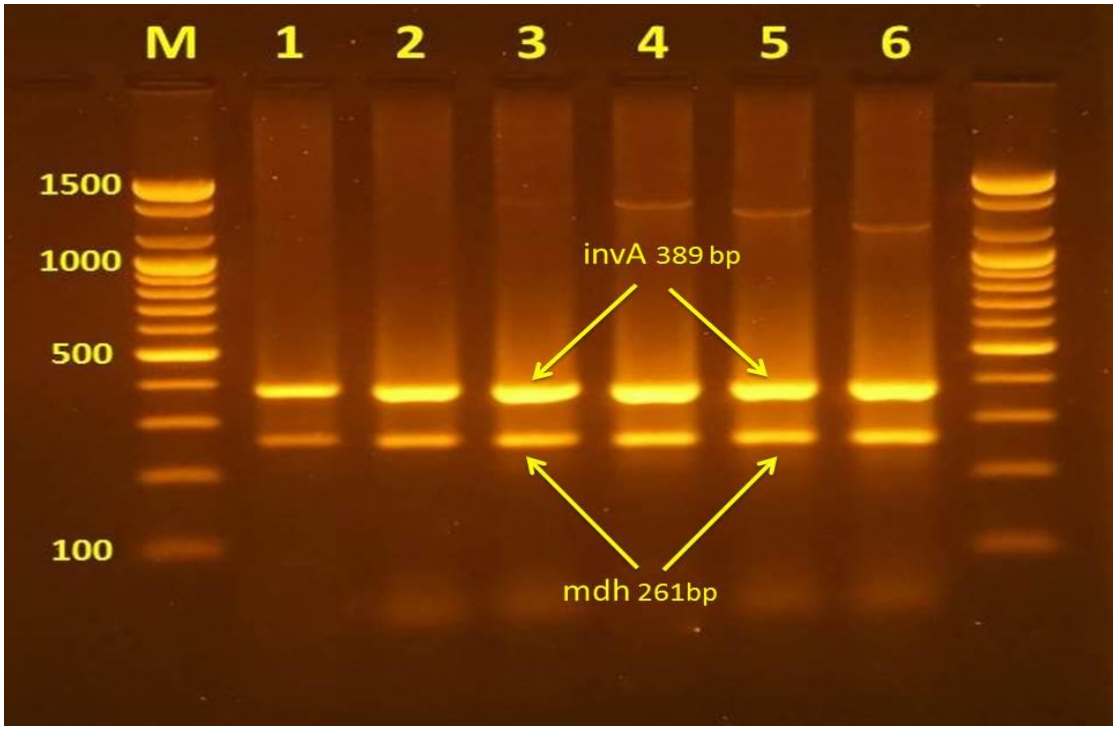

Figure 4. mPCR amplification of 389bp *Salmonella* Spp. ,261bp *Salmonella typhimurium* representative samples determined by PCR and detected by 2% agarose gel electrophoresis. Lane M: 50pb molecular size marker ladder. Lanes: 1-10 positive samples. Lane 11: negative control.

Discussion

Detection of pathogenic bacteria from 400 samples were tested by Conventional method, the results indicate 73 samples (18.25%) out of the 400 showed positive results for more than one type as shown in table (2). All kinds of food ,beverage and ice cream were contaminated with Salmonella in varying degrees with the exception of pomegranate juice and watermelon, which were not contaminated. The presence of *Salmonella* in foods and beverages could be due to several reasons such as contamination of raw material, poor hygienic conditions, contamination of water sources and unsanitary processes of foods and beverages. Frozen chicken, frozen meat, and minced meat were most polluted with *Salmonella* (Figure 1). The results indicated that meat products were the more contaminated than plant products. Compared to foods of animal origin, which are usually consumed once cooked, fruit and vegetables are mostly eaten raw and therefore a significant part of foodborne outbreaks due to the consumption of raw vegetables has been attributed to *Salmonella* (Cantoni & Bersani, 2010).

In the current study, *S. **t**yphimurium* was detected in 64% of examined frozen chicken samples. This result is higher than that reported by Abdellah *et al* (2009) who reported *Salmonella* contamination in chicken meat and giblets, 4 different serotypes were identified of which *S. typhimurium* (40.35%) was the most frequent, and Abd El-Aziz (2013) who detected *S. typhimurium* at rate of 44%, 40% and 48% in chicken meat, liver and heart, respectively, but not in gizzard. *Salmonella* spp. was analyzed in beef and chicken and in beef hamburgers, of the 80 hamburger samples analyzed, 22 (27.5%) were positive for *Salmonella* spp., 10 (12.5%) beef and 12 (15%) chicken and beef hamburgers (Fortuna *et al*., 2012). In a similar study Almeida Filho *et al*. (2006) analyzed 30 samples, of which 15 (30%) were contaminated with *Salmonella* spp. On the other hands other studies conducted to analyze *Salmonella* spp. in hamburgers did not reveal the presence of the pathogen in this food (Bezerra *et al*. ,2010).The traditional method for the detection of *Salmonella* reveal *Salmonella* and bacteria-like *Salmonella*, so that need further serological detection to distinguish the *Salmonella* spp. Traditional *Salmonella* detection methods are based on cultures using selective media and characterization of suspicious colonies by biochemical and serological tests (Ben Salem *et al*., 2010). Traditional culture-based methods for detecting Salmonella are reliable but labor-intensive and time-consuming, demanding several days for a definitive result(Amagliani *et al*., 2007 ;Kataria *et al*., 2013). Traditional approaches for analysis of *Salmonella* has relied on cultural techniques and several selective differential media have used for differentiation. However, biochemical analysis for an enzyme associated with the particular pathogenic trait could be cross reactive with other enteric bacteria. The results of serological test indicate that 61 samples (83.56 %) out of the 73 were *Salmonella* spp. ,and 13 samples out of 61 were *Salmonella typhimurium* (Table 3). Serological examination showed that the highest contamination of food with

bacteria was by *salmonella typhimurium* (30.14%) followed by *salmonella anatum* (20.55%) (Table 3).

In the current study, detection of pathogenic bacteria from 400 samples were tested by mPCR. , the results indicated that sixty one 15.25% out of 400 examined samples were positive results (Figure 3). This result is higher than that reported by Stock & Stolle(2001) and Molla *et al*.(2003) which his result (6.3%) and (12.1%)from minced meat samples, respectively, whereas lower than Hassanein *et al*.(2011)who reported that out of the total 75 meat samples examined, *Salmonella* was detected in 5 (20%) of minced frozen beef, 9 (36%) of frozen chicken leg and 13 (52%) of frozen chicken fillet samples .The method was evaluated with 1,293 naturally contaminated food samples and compared to the conventional cultural method , of 55 positive PCR samples, 45 were confirmed by the cultural method (Made *et al*., 2004). Paião *et al*. (2013) used mPCR for detection of *Salmonella,* the results showed the presence of *Salmonella* spp. in 25% of samples, *S. Enteritidis* was present in 12% of the *Salmonella*-positive samples and *S. typhimurium* in 3% of the samples. Raafat *et al*.(2011) found high relationship between isolates from chicken meat and patient with food poisoning signs indicates a close genetic relationship between *Salmonella typhimurium* isolated from poultry meat and that isolates from human. results obtained by Saeed *et al*.(2013) support this finding.

Detection of the *S*. spp. in food products by culture method is a time consuming procedure. In this study, results showed that a PCR-based method detects *S.* spp leading to a substantial reduction in analysis time. Two important steps in achieving this time reduction were the use of *in vitro* DNA amplification and the amplification of three independent gene fragments in a single multiplex PCR analysis. Multiplex PCR, therefore, allows a more rapid and cost-effective detection of more than one pathogen that may be present in a single food sample. (Liu *et al*., 2002). It is important to emphasize that PCR cannot distinguish between dead and living cells because this technique uses just DNA as template. So, it must make the PCR test in parallel with the microbiological test. Furthermore it must be considered that differences between PCR and microbiological test results may be attributable to the fact that target cells may have been injured or died despite the enrichment procedures that were non-culturable but detectable by PCR(Ben Salem *et al*., 2012).

Multiplex PCR successfully amplified the DNA fragments corresponding in size as fallows *Salmonella* spp.389 bp **(***invA* target gene**),** *Salmonella typhimurium* 261bp **(** *Mdh* target gene) (figure 4) but not from all the non-*Salmonella*. *fliC* gene did not work when used with *invA* and *Mdh* genes ,may be due to its large size and need different temperature cycles. The optimization of multiplex PCRs can counter several difficulties, including poor sensitivity or specificity and/or preferential amplification of certain specific targets (Polz & Cavanaugh , 1998). The presence of more than one primer pair in the multiplex PCR increases the chance of obtaining spurious amplification products, primarily because of the formation of primer dimers

(Brownie *et al*., 1997). These nonspecific products may be amplified more efficiently than the desired target, consuming reaction components and producing impaired rates of annealing and extension. Thus, the optimization of multiplex PCR should aim to minimize or reduce such non-specific interactions (Ben Salem *et al*., 2012). Therefore, in multiplex PCR, as more loci are simultaneously amplified, the pool of enzyme concentrations, PCR buffer constituents and nucleotides becomes a limiting factor and more time is necessary for the polymerase molecules to complete synthesis of all the products (Chamberlain *et al*., 1989). Multiplex PCR for *Salmonella* serotyping was applied using five primer sets in the same reaction mixture. Using these five STM primers with the 19 *Salmonella* serovars, four distinct groups were identified (Ben Salem *et al.* 2010). Salmonella Spp. can be detected with a multiplex-PCR (mPCR) which can amplify several target genes in one PCR run, such as *Hin, Hli* and *PhoP* genes (Ben Salem et al., 2010), *ompC , SdfI, ViaB*, and *Spy* genes (de Freitas et al., 2010), *invA*, *rfbE*, and *hlyA* genes(Suo et al., 2010), *rfbJ , fjlC, fljB, invA , spv* and *sefA* genes (Akbarmehr,2011), *Rfbj, Flic, Fljb* and *Sdf* genes (Hassanein et al., 2011). *Typh* ,*invA* genes(Kumar, 2012), *invA*, *fliC* and *sefA* genes (das Chagas et al., 2013), *InvA*, *IE 1* and *FlicC* genes (Paião et al., 2013).

Our data indicate that the Multiplex PCR test using two gene primers was as sensitive, accurate, safe and rapid as a standard culture method in detecting *Salmonella*. PCR is more reliable than conventional identification since it is based on stable genotypic characteristics rather than relying on biochemical or physiological traits, which can be genetically unstable (Lawrence & Gilmour , 1994), and because of the bacterial adaptation to different environments causing similarities in phenotype, as well as resistance to ingredients in enrichment and selective media, therefore the transition from conventional methods of detection to genetic methods should be carried out (Gouws and Liedemann, 2005) . Moreover, the presence of other organisms on XLD selective agar plates may interfere with the production of H_2S by *Salmonella* which is important for identification (Van Kessel *et a*l.,2004).

From the results of the present study, it could be concluded that *Salmonella* is widespread in different food sources in Baghdad. The mPCR technique was successfully adapted to identify *Salmonella typhimurium.* The protocols and primers used were effective for the amplification of a fragment of 389 bp of *invA* gene and a fragment of *Mdh* gene for the *Salmonella typhimurium.* Also successfully applied a conventional thermocyclers for rapid multiplex PCR detection of *Salmonella* within a much shorter time than conventional PCR . The specificity and sensitivity were comparable to the currently used standard culture method. Results of this study can be used by agriculture and health organizations in Iraq.

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