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Molecular identification of *Salmonella entrica* serovars in ready-to-eat fast foods using multiplex-PCR

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Key words: Ready-to-eat (RTE) fast foods, Multiplex PCR, *Salmonella entrica* Serovars Typhimurium and Enteritidis.

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Abstract

Foodborne pathogens are becoming a globally formidable health complication and perceived as a major health concern in the Kingdom of Saudi Arabia (KSA). In our previous study, 23 of *Salmonella entrica* were isolated from ready-to-eat (RTE) fast foods included shawarma, falafel, vegetable salad and kibtha, from fifteen different food corners in Al-Quwayiyah, Riyadh Region of Saudi Arabia. Identification was based on conventional culture, biochemical and serological techniques. The objective of the current study is to confirm identification the serovars of *S. entrica* isolates using multiplex-PCR. For this purpose, two specific oligonucleotide primer pairs were used to amplify *flic and sefA* genes for *S.entrica* serovars Typhimurium and Enteriditis. The results revealed that 5 of *S.* Typhimurium and 3 of *S.* Enteriditis were obtained from 8 positive *Salmonella* spp. In shawarma samples, 2 of *S.* Typhimurium and 1 of *S.* Enteriditis were obtained from 3 positive *Salmonella* spp. In falahfel samples, 6 of *S.* Typhimurium and 2of *S.* Enteriditis were obtained from 8 positive *Salmonella* spp. In vegetable salad samples, only 2 of S. Typhimurium and 2 of *S.* Enteriditis were obtained from 4 positive *Salmonella* spp. in Kibtha samples. These results highlight the importance of the multiplex PCR over serotype for the rapid detection of *Salmonella* from RTE fast food samples.

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Introduction

In today's world the fast food industry has grown very fast because of busy and hectic life schedule has opened of the people. Ready-to-eat (RTE) fast food is one of the most liked and preferred includes eaten shawarma, falafel, vegetable salad and kibtha sold in fast food restaurants in Saudi Arabia. Consumption of refined meat is appreciably large in Saudi Arabia, especially is chicken. Traditionally, lamb, goat and camel meat had been customary in the diets of Saudis and European Commission EC (European Commission, 2017).

Foodborne diseases endure a major problem in developing countries because of lack of personal hygiene and food safety measures. As much as 70% of diarrheal diseases in developing countries are believed to be of foodborne origin (Amany *et al.,* 2013).

There are many incidences of foodborne diseases which have been reported in different cities of Saudi Arabia. Recent studies in sulyyel, Riyadh, showed that people often complained of gastritis 21 hours after having the lunch/dinner in the marriage party was due to *Salmonella* spp. (Khalil Mohamed *et al.,* 2017).

Foodborne pathogens were recently reported of high morbidity in Hail and Abha, 39 cases of *Staphylococcus aureus* and 26 cases of *Salmonella enteritidis* respectively (Khalil Mohamed *et al.,* 2017). Although foodborne diseases do not always result in acute gastroenteritis, food represents an important vehicle for pathogens causing acute gastro gastroenteritis. Diarrheal diseases are the commonest manifestation of food poisoning and in some cases, it is very lethal too.

Conventional culture methods have traditionally been considered as the “gold standard” for the isolation and identification of foodborne bacterial pathogens. They consist of a series of steps that include nonselective enrichment, selective enrichment, selective/differential plating and, finally, morphological, biochemical and serological confirmation. This standardized classical culture method is known to be sensitive and inexpensive, but culture methods are labor-intensive and time-consuming, because they require at least, three working days to produce a negative result and five to ten working days for confirmed positive results. Moreover, due to environmental factors, variations in gene expression of microorganisms can occur and may affect the results of biochemical tests. Furthermore, viable but non cultivable cells are not detected by the conventional methodology. Rapid methods for the detection of *Salmonella* in food have been developed, for example, electrical techniques, immunoassays and nucleic acid probe analyses, but there are still problems with their sensitivity and specificity (Omar B Ahmed *et al.,* 2014).

Salmonella enterica serovars Enteritidis and Typhimurium which are represent the most predominant isolated organisms in most cases associated with the consumption of contaminated poultry, pork and beef products (Doaa *et al.,* 2013).

In recent years, PCR-based methods have been reported as a rapid, specific and sensitive alternative, and have been increasingly used to identify several microbial species from foods (JaroslavPochop *et al.,* 2013). PCR has become an important detection tool for pathogens in foods; PCR can also identify strain differences by targeting gene(s) or sequences exhibiting polymorphisms or variability in its distribution within the bacterial population (Mahmoud Elhariri *et al.,* 2017).

In our recently study (Samir A. Alharbi *et al.,* 2019), 23 *S. entrica* were isolated from 155 RTE fast food samples i.e. shawarma, falafel, vegetable salad and Kibtha collected from different 15 restaurants at Al-Quwayiyah city revealed that, KSA.

These isolates were morphologically, biochemically and serologically identified. Therefore, the objective of this study is to identify the serovars of the 23 *S. entrica* using multiplex PCR.

Materials and methods

DNA extraction

Isolates of 23 *Salmonella entrica;* which identified using conventional culture, biochemical and serological techniques in our previous study (Samir A. Alharbi *et al.,* 2019), were grown in 10 ml Xylose Lysine Desoxycholate (XLD) at 37°C for 24h. The overnight cultures were centrifuged at 3000rpm for 5 min and the supernatant were decanted carefully. The bacterial pellets were washed three times with phosphate buffer saline pH 7.2 and resuspended in 400µl tris-EDTA buffer (pH 8.0) and heated in water bath at 100°C for 20 min. There were left to cool at room temperature and centrifuged at 14,000rpm for 10 min. An aliquot of 5µl of the supernatant was used as template DNA in the multiplex PCR (Moussa IM *et al.,* 2010).

Oligonucleotide primers

Three specific oligonucleotide primer pairs were used for multiplex PCR. The first was ST11 to ST15 primers were selected which amplify fragment of 429 bp that is specific *Salmonella* spp. (LailaNimri *et al.,* 2014). The second was Fli15-Tym primers were specific for the *fliC* genes of *S.* Typhimurium to produce amplicon size of 559 bp (Moussa IM *et al.,* 2012). The third was Sef167 to Sef478 primers specific for the *sefA gene* found in *S.* Enteritidis which amplify fragment of 312 bp. All specific primers were synthesized by Sigma in Germany according to (Hannan A, *et al.,* 2014).

DNA amplification

PCR amplifications were performed in a final volume of 50 µl in micro-amplification tubes. The reaction mixtures consisted of 5 µl of the DNA template, 5 µl 10X PCR buffer (75mM Tris-HCL, pH 9.0, 2mM $MgCl_2$, 50mM KCl, 20mM $(NH_4)_2SO_4$), 1µl dNTPs (40µM), 1µl (1U Ampli Taq DNA polymerase) 1µl (25pmol) from the forward and reverse of three sets of primers and the volume of the reaction mixture was completed to 50µl using deionized distilled water. The thermal cycler (C1000 Touch™ thermal cycler (CFX96, BIO-RAD, USA)) was adjusted according to (Moussa IM *et al.,* 2012) as follows: initial denaturation at 94°C for 5 min, followed by 35 cycles of (denaturation at 94°C for 1min, annealing at 56°C for 1min and extension at 72°C for 1min). Final extension was carried out at 72°C for 10 min.

Agarose gel electrophoresis

Ten microliters of the PCR products as well as 100 bp molecular marker (Sigma, Germany) were loaded into 1% agarose gel containing 1 µg of ethidium bromide per ml and DNA bands were captured and images were analysed with the Molecular Analyst/PC Software (Bio-rad, USA) from the Bio-rad Universal Hood II Systems for Gel Doc ™ and ChemiDoc™ Imaging systems.

Results

Confirmation of the Salmonella isolates using Multiplex PCR

Multiplex PCR was used to confirm the serovars of 23 *Salmonella entrica*. Using primers specific for genus *Salmonella*, for *S.* Enteriditis (*sefA* gene) and *S.* Typhimurium (*fliC* gene) serovars. All positive *Salmonella* serovars samples for amplification of 429 bp fragments, Furthermore, *S.* Typhimurium and *S.* Enteriditis isolates were positive for amplification of 559 bp and 312 bp shown in (Table 1) respectively.

Table 1. Primer used for the multiplex PCR detection of *Salmonella* Typhimurium and Entriditis serovars.

Target	Primer sets	sequence 5'→3'	Size (bp)
Salmonella genus (Random sequence)	ST11(F) ST15 (R)	GCCAACCATTGCTAAATTGGCGCA GGTAGAAATTCCCAGCGGGTACTGG	429
*S.*Typhimurium (*flic* gene)	Fli15(F) Tym (R)	CGGTGTTGCCCAGGTTGGTAAT ACTCTTGCTGGCGGTGCGACTT	559
S. Entriditis *(sefA gene)*	Sef167(F) Sef478(R)	AGGTTCAGGCAGCGGTTACT GGGACATTTAGCGTTTCTTG	312

F: forward primer

R: reverse Primer

The results revealed that 5 of *S.* Typhimurium and 3 of *S.* Enteriditis were obtained from 8 positive *Salmonella* spp. in shawarma samples (Fig. 1), 2 of *S.* Typhimurium and 1 of *S.* Enteriditis were obtained from 3 positive *Salmonella* spp. in falahfel samples as shown in (Fig. 2). Fig. 3 shows 6 of *S.* Typhimurium and 2 of *S.* Enteriditis were obtained from 8 positive *Salmonella* spp. in vegetable salad samples, while only 2 of *S.* Typhimurium and 2 of *S.* Enteriditis were obtained from 4 positive *Salmonella* spp. in kibtha samples as shown in (Fig. 4) and Incidence of 23 *Salmonella* Serovars by using Multiplex PCR and shown in (Table 2) respectively.

Table 2. Incidence of 23 *Salmonella* Serovars by using Multiplex PCR.

Identified serovars of *S. entrica*	RTE fast food samples				Total	
	Fig.1	Fig. 2	Fig. 3	Fig. 4		
	Shawarma	Falahfel	Vegetable salad	Kibtha		
	No	No	No	No	No	%
S. Typhimurium	5	2	6	2	15	65%
S. Enteritidis	3	1	2	2	8	35%
Total	8	3	8	4	23	100%

Discussion

The present data revealed that 23 *Salmonella* isolates from RTE fast food samples such as shawarma, falafel, vegetable salad and Kibtha. It is clear 15 *S.* Typhimurium and 8 *S.* Enteritidis were identified serologically with incidence of 65.22% and 34.78% respectively, lower results reported by (Dhaher FH *et al.,* 2011). The two most commonly identified causative agents of food borne salmonellosis are *Salmonella enterica* serotypes Typhimurium and Enteritidis (Galis AM *et al.,* 2013). Both serotypes have the ability to colonize the reproductive organs of hens and are major causes of food borne illness (Whiley H *et al.,* 2015).

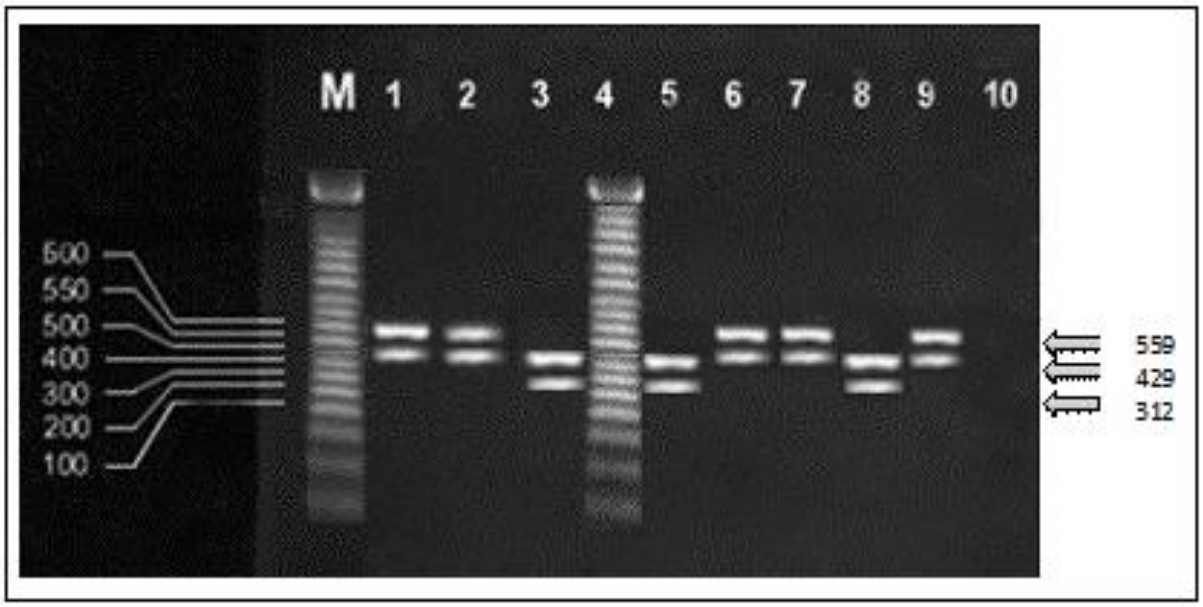

Fig. 1. Multiplex PCR on Agarose gel electrophoresis showing amplification of 429, 559 and 312 bp fragments from the extracted DNA of Salmonella isolates from shawarma. Lane M: 100 bp DNA marker, Lanes 1, 2, 6, 7 and 9: positive amplification of 559 bp fragment of S. Typhimurium isolates, Lanes 3, 5 AND 8: positive amplification of 312 bp fragments of S. Enteritidis isolates, Lane 4: positive control *S.* Typhimurium (ATCC 14028), Lanes 10 negative control (distilled sterile water).

Salmonella is detected by standard bacteriological, biochemical and serological tests. These tests are generally time-consuming, tedious and costly as they require hundreds of antisera as well as well trained technicians (Nori EM *et al.,* 2010). The Direct-PCR will reduce the time required for the decision about the *Salmonella* positive samples. Amplification of DNA sequences unique to an organism utilizing the PCR enhances both the detection speed and the level of sensitivity at which organisms can be distinguished and has been increasingly used to identify several bacterial species from food and clinical samples (Mahmoud Elhariri *et al.,* 2017).

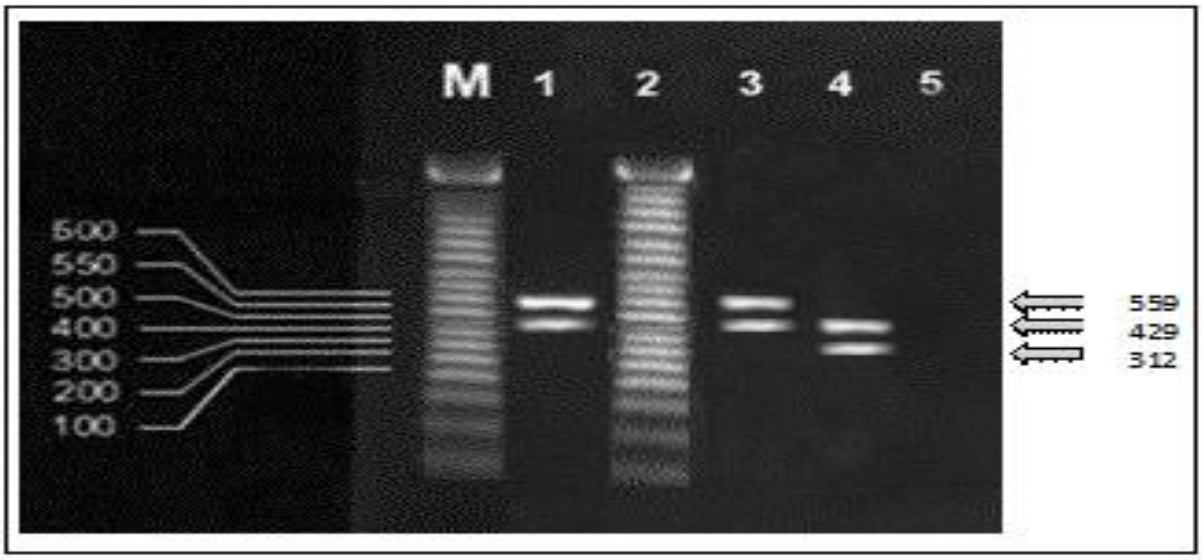

Fig. 2. The specific primer after Multiplex PCR on Agarose gel electrophoresis showing amplification of 429, 559 and 312 bp fragments from the extracted DNA of Salmonella isolates from falahfel. Lane M: 100 bp DNA marker, Lanes 1, 3 positive amplification of 559 bp fragment of S. Typhimurium isolates, Lanes 4 positive amplification of 312 bp fragments of S. Enteritidis isolates, Lane 2: positive control S. Typhimurium (ATCC 14028) , Lanes 5 negative control (distilled sterile water).

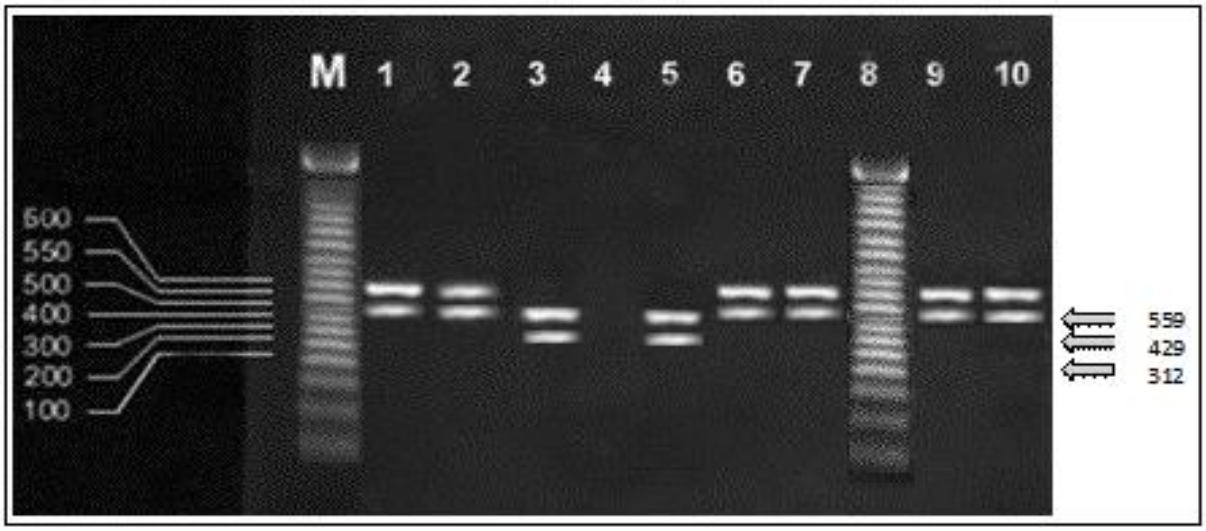

Fig. 3. The specific primer after Multiplex PCR on Agarose gel electrophoresis showing amplification of 429, 559 and 312 bp fragments from the extracted DNA of Salmonella isolates from vegetable salad. Lane M: 100 bp DNA marker, Lanes 1, 2, 6 and 7, 9, 10: positive amplification of 559 bp fragment of S. Typhimurium isolates, Lanes 3, 5: positive amplification of 312 bp fragments of S. Enteritidis isolates, Lane 8 : positive control S. Typhimurium (ATCC 14028) , Lanes 4 negative control (distilled sterile water).

The results revealed that 15 (65%) of *S.* Typhimurium and 8 (35%) of *S.* Entriditis were most frequent among the total 23 *Salmonella* isolates. The total of 23 *Salmonella* isolates were studied by bacteriological analysis, biochemical, serological and confirmed by Multiplex PCR.

All isolates were subjected to *Salmonella entrica* random sequence (ST11, ST 15) and were confirmed as *Salmonella* positive by the predicted product of 429 bp DNA fragments. The results obtained in the present study were in accordance with (Laila Nimri *et al.,* 2014; Moussa IM *et al.,* 2012; Jakeen EI Jakee *et al.,* 2016). The ability of *Salmonella* specific primers to detect *Salmonella* species rapidly and accurately in the present study is primarily due to the primer sequences that are selected from the gene random sequence (ST11, ST 15) and *flic* of *S.* Typhimurium showed amplification of 429bp and 559bp fragments as reported (Doaa MA *et al.,* 2013; Moussa IM *et al.,* 2012; Jakeen EI Jakee *et al.,* 2016) While all *S.* Entriditis showed amplification of 429bp and 312bp fragments using *Salmonella* spp. random sequence (ST11, ST 15) and *sefA*. The results obtained in the present study were in accordance with (Moussa IM *et al.,* 2012; Jakeen EI Jakee *et al.,* 2016).

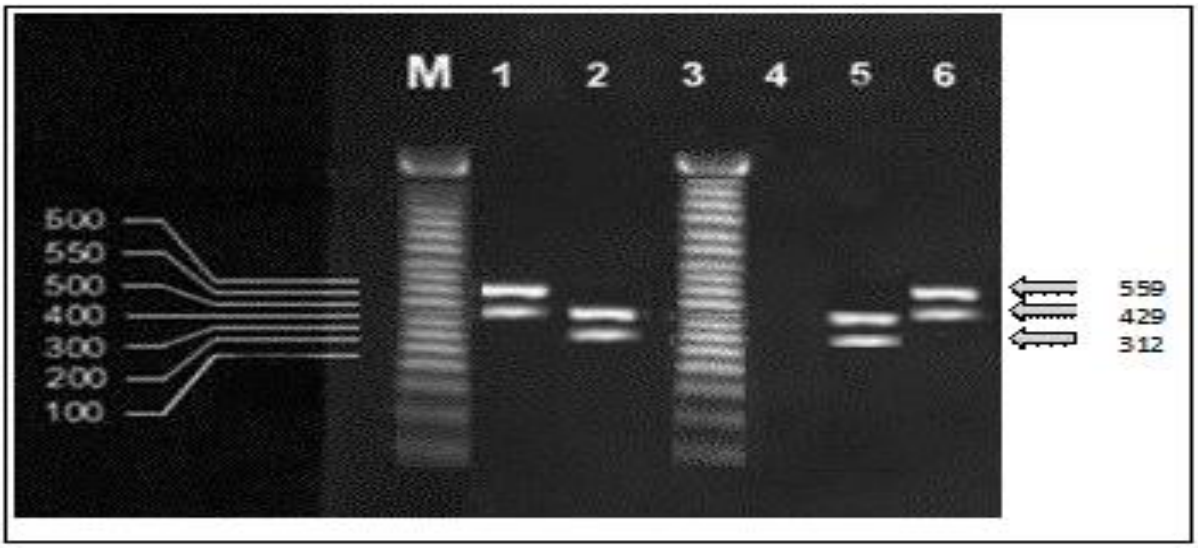

Fig. 4. The specific primer after Multiplex PCR on Agarose gel electrophoresis showing amplification of 429, 559 and 312 bp fragments from the extracted DNA of Salmonella isolates from liver. Lane M: 100 bp DNA marker, Lanes 1 and 6: positive amplification of 559 bp fragment of S. Typhimurium isolates, Lanes 2 and 5 : positive amplification of 312 bp fragments of S. Enteritidis isolates, Lane 3: positive control S. Typhimurium (ATCC 14028) , Lanes 4 negative control (distilled sterile water).

These results highlight the importance of the multiplex PCR over serotype for the rapid detection of *Salmonella* from RTE fast food samples. To best our knowledge, this is the first multiplex PCR assay to simultaneously detect *Salmonella* genus, *Salmonella* subsp. *S.* Typhimurium and *S.* Entriditis from RTE fast foods.

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Conflict of interest statement

We declare that we have no conflict of interest.

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