j.Egypt.vet.med.Assoc 83, no 1. 61 – 73 (2023)

MULTIPLEX PCR FOR DETECTION OF SALMONELLA ISOLATES FROM READY TO EAT FOODS IN EGYPT

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ABSTRACT

Salmonellae are the main food-borne pathogens that cannot lead to inescapable burden, however they produce the possibility of proximity in the gastrointestinal tract leading to gastroenteritis. *Salmonella* infection may take a septicemic form leading to bacteremia and gastroenteritis. In this study, 100 samples were collected from ready to eat foods of beef and chickens: burger, kofta, chicken, and sausages. Samples were examined using a PCR amplification assay where the virulence gene *inv*A was targeted and PCR was more effective in the detection and identification of *Salmonella* isolates. Our study's objective was to develop a technique for detecting and categorizing *Salmonella* utilizing the dominant class five serovars. By using multiplex PCR, Gallinarum Pullorum, *Salmonella* enteritidis, *Salmonella enterica* ser. Typhimurium typhi, ser. of *Salmonella enterica* Dublin, and ser. of *Salmonella enterica* were identified distinctly.

Keywords:

Salmonella, PCR, multiplex PCR, Ready-to-eat food.

INTRODUCTION

Salmonella is a bacterium that commonly causes food borne illness, sometimes called (Food poisoning). *Salmonellae* live in the intestines of human and many animals.

Salmonella is usually transmitted to people through consumption of food contaminated with the bacteria like: eggs and egg products, milk and dairy products, meat and poultry products as well as through tap water **(Bailey, 1998).**

Fresh poultry and meat are commonly putrefied because of their enriched nutrient component, water activity (0.98 to 0.99), and near neutral pH (5.5 to 6.5), which is the optimal environmental condition for *Salmonella.*

Bacteria present in dirt and fecal material associated with slaughter and evisceration areas could contaminate poultry and meat. In the presence of a contaminated carrier during processing, the

quality of the final product could be threatened by *Salmonella*. The increasing demand for chicken products may have the unintended consequence of the increased risk of chicken-associated 6 food illnesses. In turn, hygiene management should be greatly considered during processing. *Salmonella* easily forms a biofilm on food contact surfaces **(Reij *et al.,* 2004; Rodrigues *et al.,* 2011 and Møretrø *et al.,* 2012).**

Once a biofilm is formed, it protects the embedded bacteria from external physical and chemical treatment **(Milanov *et al.,* 2009 and Ashrafudoulla *et al.,* 2021).**

Although antibiotics are not generally used to treat human *salmonella* infections, they may be lifesaving in infections or in immune-compromised patients. For this reason, maintaining the susceptibility of *Salmonella* to clinically-imported antibiotics is imperative.

Salmonellae are divided into two types (*Salmonella* Bongori and *Salmonella* Enterica). *Salmonella enterica* is divided into subspecies (*Salmonella entrica* subspecies Entrica, *Salmonella* entrica subspecies Salamae, *Salmonella entrica* subspecies Arizonae, *Salmonella entrica* subspecies Diarizonae, *Salmonella entrica* subspecies Houtenae and *Salmonella entrica* subspecies indica) **(Althouse *et al.,* 2003 and Battistuzzi *et al.,* 2004).**

Salmonella enterica subspecies Entrica is mostly isolated in the majority of cases of salmonella infection so it is subdivided into Typhoidal type (*Salmonella* Typhi and *Salmonella* Paratyphi - Cause typhoid fever / Non Typhoidal type - (*Salmonella* Typhimurium and *Salmonella* Enteritidis cause food poisoning). According to the Centers for Disease Control and Prevention (CDC), non-typhoidal *Salmonellosis* causes about 1.35 million illnesses, 26,500 need hospitalizations, and 420 deaths in the USA every year **(CDC, 2019)**. In Korea, a large outbreak of *S.Thompson* infections caused by contaminated eggs led to 3516 patients hospitalized in 2019 **(MFDS, 2019).** According to the FAO, 20% of poultry products in the world are contaminated with *Salmonella*, and they can persist for a long time in the animal facilities because they can form a surface film **(Vestby *et al.,* 2009).**

Salmonella Typhimurium and *Salmonella* Enteritidis are the serovars commonly causing human infection, while *Salmonella* Typhimurium are mostly associated with poultry and bovine meat consumption.

The estimated total cost associated with *Salmonella* incidences may be up to several billion dollars annually **(Frenzen *et al.,* 1999 and WHO, 2005).**

Salmonella may be found and identified using conventional techniques that are labor- and time-intensive and depend on selected media. These limitations may be supplemented by

cutting-edge molecular biology techniques like a multiplex PCR experiment. The pathogen's kind is a crucial factor in the diagnosis.

The purpose of the current study was to establish a test system based on polymerase chain reaction that would likely identify and type some important members of the genus *Salmonella* (*Salmonella enterica* ser.Enteritidis,*Salmonella enterica* ser.Typhimurium,*Salmonella enterica* ser. Typhi, *Salmonella enterica* ser. Dublin, and *Salmonella enterica* ser. Gallinarum-Pullorum).

MATERIAL AND METHODS

1. Samples collection and preparation:

One hundred ready-to-eat meals were gathered from a variety of fast food chains and street vendors with varying degrees of hygiene. Samples included beef burger, kofta, sausages, and chicken (25 of each). Each collected sample was placed in sterile plastic bag, sealed, labelled, and maintained in an ice box before being moved as soon as possible to the laboratory under strict aseptic conditions. At the laboratory, the content (Meat part) of each sample was aseptically and carefully removed and mixed well in a sterile mortar. (APHA, 2001).

Isolation and identification of *Salmonella* in each one was detected.

2. Isolation of *Salmonella* spp:

2.1. Pre-enrichment in a Non- selective liquid medium.

Twenty five grams of each prepared sample were chopped into small pieces with sterile forceps and scissors, and blended for two minutes in a sterile blender jar containing 225 ml of 0.1% sterile buffered peptone water (BPW) as a pre-enrichment broth as advised by **Edel and Kamplmacher (1973).** Each blended sample was aseptically transferred to a 500 ml sterile wide-mouth, screw-capped jar before being incubated at 37 °C for 18 ± 2 hrs.

2.2. Enrichment in a selective liquid medium:

A volume of 0.1 ml of pre-enrichment culture was transferred into a sterile tube containing 10 ml of Rappaport Vassiliadis broth (RVS) and the tubes were then vortexed and incubated at 41.5°C ± 1for 24hrs. **(Rappaport *et al.*, 1956 and Harvey and Price, 1981).**

2.3. Selective plating:

(XLD) was used. A loopful of each incubated tube was streaked onto xylose lysine desoxychoclate agar (XLD) plate and incubated at 37°C for 24hrs. After incubation, the plates were examined for the presence of typical pink colonies of salmonella with or without black

centers. The suspected colonies were subcultured onto nutrient agar plates and incubated at 37°C for 24 hours. The purified suspected colonies were selected and streaked onto nutrient agar slopes for further identification. The purified isolates were identified morphologically, biochemically and serologically.

3. Identification of *Salmonella* spp:

Films of pure suspected cultures were stained using Gram's Method and examined microscopically under oil emersion lens. *Salmonella* are 2-3 µm, pink to red Gram-negative short bacilli with rounded end **(Cruickshank *et al.*, 1975)**.

Confirmatory biochemical tests were applied on the suspected *Salmonella* isolates according to **McFaddin (2000).** On Triple Sugar Iron (TSI) agar, suspected isolates were stabbed into the bottom of the butt with a needle, and then it was drawn over the slant, for production of a sufficient surface growth. The inoculated tubes were incubated at 37°C for 24-48 hours. Hydrogen sulphide (H_2S) production was noted by blackening the medium, while acid formation was indicated by yellow color. Also, the ability of the isolates to utilize citrate for energy was tested on Simmon's citrate agar tubes incubated at 37°C for 48-96 hours. The development of blue coloration indicated utilization of citrate where *Salmonellae* utilize citrate. In addition, Christensen's agar medium was inoculated with the suspected isolates and incubated at 37°C for 24 hours. Development of pink color denoted a hydrolysis of urea. Negative tubes were re-examined after further incubation for 24 hours. *Salmonellae* are negative for urea hydrolysis.

4. Serological identification and serotyping of *Salmonella* isolates:

Isolates proved biochemically to be *Salmonella* were subjected to serological identification according to Kauffman -White scheme **(Kauffmann, 1974).**

5. PCR amplification reaction for *Salmonella*-specific gene (*inv*A)

Bacterial DNA was extracted using the QIA amp kit as described by **Shah *et al*. (2009).** The PCR cycling protocol (Thermal cycler) was applied as following: Initial denaturation at 94°C for 60 seconds. Denaturation at 94°C for 60 second, Annealing at 64°C for 30 second. For 35 cycles, Extension at 72°C for 30 second, Followed by a final extension at 72°C for 7 min **(Shanmugasamy *et al*., 2011).**

6. Multiplex PCR:

A multiplex PCR assay was conducted for simultaneous identification of five core *Salmonella* members (*Salmonella enterica* ser. Enteritidis, *Salmonella enterica* ser. Typhimurium,

Salmonella enterica ser. Typhi, *Salmonella enterica* ser. Dublin, *Salmonella enterica* ser. And Gallinarum-Pullorum). Primer sets used in the assay as well as the expected products are depicted in (Table 1). Amplification was run at initial denaturation at 94oC for 2 min followed by 40 cycles of denaturation- 94ºC for 30s; annealing at 63º C for 30s and extension 72ºC 40s with Final extension at 72º C for 5 min. PCR products were loaded into 1% agarose gel in TBE buffer containing ethidium bromide followed by visualization on a UV trans illuminator **(Surzycki, 2000).**

Table (1): Nucleotide sequences of the primers utilized in the *Salmonella* PCR assays and the suspected products' sizes.

Target	Primer	5*-3*	PCR product, bp.
Salmonella spp.	Salm 3	GCTGCGCGCGAACGGCGA	387
	Salm4	TCCCGCCAGAGTTCCCATT	
Salmonella enterica ser. Enteritidis	Sent F	AAATGTGTTTTATCTGATGCAAGAGG	299
	Sent R	GTTCGTTCTTCTGGTACTTACGATG	
Salmonella enterica ser. Typhimurium	Styp F	CCCCGCTTACAGGTCGACT	433
	Styp R	AGCGGGTTTTCGGTGGTTG	
Salmonella enterica ser. Typhi	Styphi_F	CACGCACCATCATTTCACC	738
	Styphi_R	AACAGGCTGTAGCGATTTAG	
Salmonella enterica ser. Dublin	Sdub_F	ACGCGAAATCTGATGGTCT	203
	Sdub_R	GCCCACCAGTTGTGAAAGG	
Salmonella enterica ser. Gallinarum Pullorum	Sgal_F	CCGCACAACACATCAGAAAG	97
	Sgal_R	AGCTGCCAGAGGTTACGCT	

RESULTS

1. Prevalence of *Salmonella* in the examined samples:

As depicted in (Table 2), out of 100 samples, 25 resulted in isolation of *Salmonella*-like isolates according to the cultural characters. Of 25 samples of each type (Beef burger, kofta, sausage, and chicken), 8, 6, 4 and 7 *Salmonella*-like isolates were obtained with percentages of 32%, 24%, 16% and 28%, respectively, with an overall incidence of 25%.

However, *salmonella* isolates as confirmed biochemically were 3, 2, 1 and 4 from different samples, respectively (Table 3).

Table (2): Positive suspected *Salmonella* isolates from different samples.

Samples	No. of collected samples	Positive samples	
		No.	%
Beef burger	25	8	32
Kofta	25	6	24
Sausages	25	4	16
Chicken	25	7	28
Total	100	25	25

Table (3): Biochemical identification for suspected *Salmonella* isolates.

Samples	No. of isolates for biochemical identification	Biochemically + ve isolates	
		No.	%
Beef	8	3	37.5
Kofta	6	2	33.3
Sausages	4	1	25
Chicken	7	4	57.1
Total	25	10	40

2. PCR assay for detection of *Salmonella Inv*A *gene*:

Isolates identified as *Salmonellae* biochemically were all positive with the PCR assay targeting the *Inv*A gene as evidenced by obtaining the suspected specific 389 bp product size (Table 4) and Fig.(1).

Table (4): PCR assay on the *Salmonella Inv*A gene in the biochemically defined *Salmonella* isolates.

Samples	Biochemically +ve isolates	PCR +ve samples	
		No.	%
Beef burger	3	3	100
Kofta	2	2	100
Sausages.	1	1	100
Chicken	4	4	100
Total	1	10	100

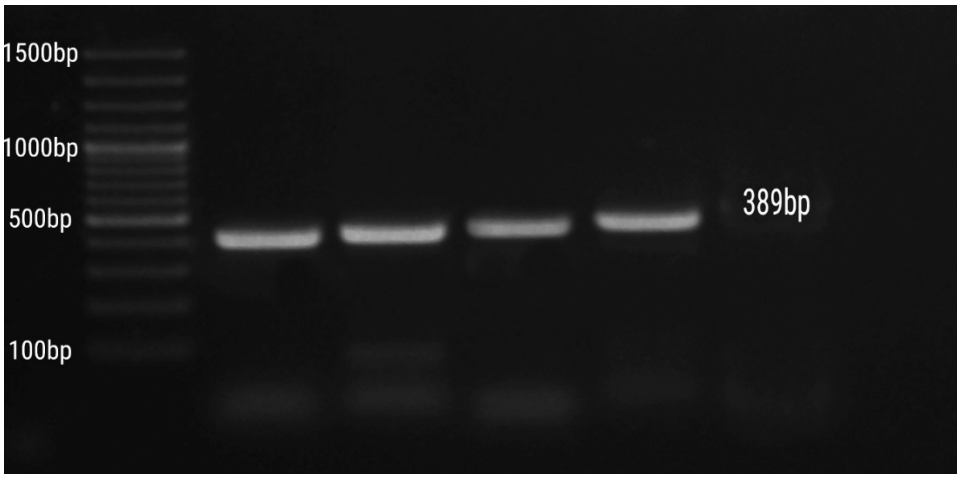

Fig. (1): Agarose gel electrophoresis of PCR of *inv*A gene (389 bp) Lane M: 100 bp ladder as molecular size DNA marker. Lane 1: Control positive *Salmonella* for *invA* gene. Lanes 2, 3 & 4: Positive *Salmonella* spp. strains for *invA* gene

B- Multiplex PCR assay for detection of different *Salmonella* spp. of serovars. As shown in (Table 5), Fig. (2), out of 3 *Salmonella* isolates recovered from beef kofta, 2 were identified as *Salmonella enterica* ser. Enteritidis and 1 was *Salmonella enterica* ser. Typhimurium by multiplex PCR. Concerning other types of samples, 2 isolates from kofta were found to be *Salmonella enterica* ser. Enteritidis and *Salmonella enterica* ser. Dublin, 1 isolate from sausage was *Salmonella enterica* ser. Enteritidis, while the 4 isolates recovered from chicken samples were categorized as 1 *Salmonella enterica* ser. Typhi, 1 *Salmonella enterica* ser. Dublin and 2 *Salmonella enterica* ser. Gallinarum Pullorum. The most common type was *Salmonella enterica* ser. Enteritidis (4), followed by *Salmonella enterica* ser. Dublin, and Salmonella enterica ser. Gallinarum Pullorum (2 each) and *Salmonella enterica* ser. Typhimurium and *Salmonella enterica* ser. Typhi (1 beach).

Table (5): *Salmonella* typing by multiplex PCR ne.

	Beef burger	Kofta	Sausages	Chicken	TOTAL
Salmonella spp.	**3**	**2**	**1**	**4**	**-**
***Salmonella enterica* ser. Enteritidis**	**2**	**1**	**1**	**-**	**4**
***Salmonella enterica* ser. Typhimurium**	**1**	**-**	**-**	**-**	**1**
***Salmonella enterica* ser. Typhi**	**-**	**-**	**-**	**1**	**1**
***Salmonella enterica* ser. Dublin**	**-**	**1**	**-**	**1**	**2**
***Salmonella enterica* ser. Gallinarum Pullorum**	**-**	**-**	**-**	**2**	**2**
Total	**3**	**2**	**1**	**4**	**10**

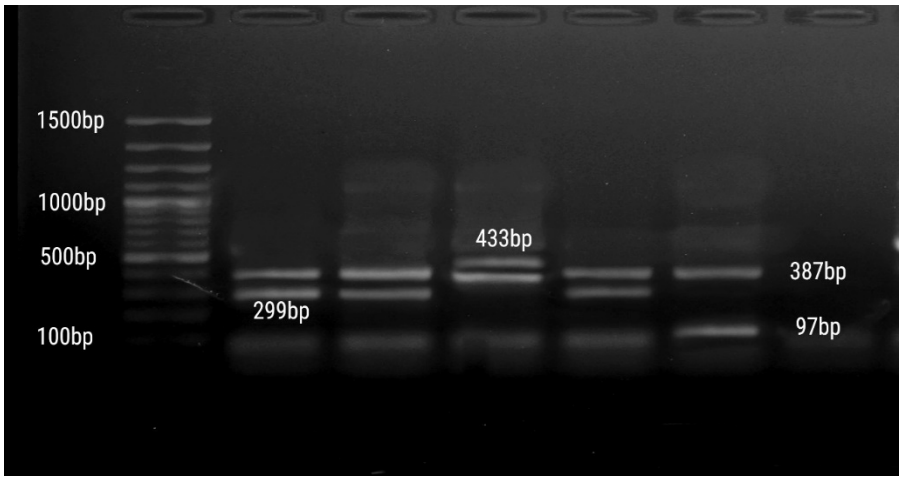

Fig. (2): Agarose gel electrophoresis of multiplex PCR of *Salmonella* spp.

Lane M: 100 bp ladder as molecular size DNA marker.

Lane 1: posiive for *Salmonella* spp. (387 bp) and *Salmonella enterica ser.* Enteritidis (299 bp).

Lane 2: posiive for *Salmonella* spp. (387 bp) and *Salmonella enterica ser.* Enteritidis (299 bp).

Lane 3: posiive for *Salmonella* spp. (387 bp) and *Salmonella enterica ser.* Typhimurium (433 bp).

Lane 4: posiive for *Salmonella* spp. (387 bp) and *Salmonella enterica ser.* Enteritidis (299 bp).

Lane 5: posiive for *Salmonella* spp. (387 bp) and *Salmonella enterica ser*. Gallinarum- Pullorum (97 bp).

DISCUSSION

There have been reports of food-borne illnesses linked to the consumption of ready-to-eat foods everywhere in the globe **(FAO, 1988; Garcia *et al.,* 2004; Chumber *et al.,* 2007 and Ghosh *et al.,* 2007).** Human incidences of food illness or gastroenteritis have been linked to ready-to-eat food **(Eley, 1996).**

According to (Table 2) findings, out of 100 ready-to-eat meat items, 25 isolates were identified as *Salmonella* (25%) according to the cultural characteristics on XLD agar. XLD agar was highly effective for isolation of *Salmonella* from clinical samples in the first place. This could be referred to components as Xylose and Lysine that are present in XLD agar. All enteric microbes except *Shigella* spp. and *Providencia* spp. ferment xylose.

Salmonella spp. is recognized by the addition of lysine as it decarboxylates lysine as xylose is depleted, resulting in alkaline conditions that give the colonies their red hue. Our findings were in line with those of **Isenberg (2004)**, who noted the great effectiveness of XLD agar in the main isolation of *Salmonella* spp. and *Shigella* spp.

The results shown in (Table 2) showed that, despite the organisms being killed by suitable cooking temperatures, their existence in ready-to-eat meat and meat products could be due to highly contaminated raw materials, inadequate heating during cooking, and post-cooking contamination, most likely by the use of infected utensils or through contaminated worker hands. Cross contamination between raw and already prepared meals is regarded as a major cause of post-cooking contamination at the same time **(National Academy of Science, 1985).**

The findings in (Table 3) showed that only 10 out of 25 *Salmonella* isolates were found to be true *Salmonellae using* biochemical processes, with a percentage of (40% of the isolates and 10% of the samples). Of the *Salmonella* 10 positive samples, 3 were beef burger samples, 2 were kofta samples, 1 was sausage sample and 4 were chicken samples.

The use of PCR to amplify DNA sequences specific to an organism increases the sensitivity and speed of the detection of those species **(Buffone *et al.,* 1991 and Ramamurthy *et al.,* 1993)** Sequences specific to the *inv*A gene found on the chromosome of all known serovars of *Salmonella* spp. include a specific gene that has been recognized as an international standard and is appropriate for PCR for the quick, less costly, and sensitive identification of this species **(Rahn *et al.,* 1992).**

In several clinical samples, a quick technique using primers for the *inv*A gene was suggested by **Lampel *et al.* (2000) and Ferretti *et al.* (2001).** This method permitted the identification of *Salmonella* serotypes within a maximum of 12 hours. All the 10 isolates tested in this investigation comprising several serotypes had the *inv*A gene operon. According to **Das *et al.* (2012),** the *inv*A gene might be employed as a particular flag gene for the quick identification of salmonella isolates from multiple sources.

Multiplex PCR is the practice of amplifying several DNA sequences concurrently using polymerase chain reaction (as if executing many distinct PCR reactions all at once in one reaction). Using numerous primers and a temperature-mediated DNA polymerase in a thermal cycler, this procedure amplifies DNA in samples. To enable all primer pairs to function during PCR at the same annealing temperature, the primer designs for each pair must be tuned. A single PCR mixture is used in multiplex PCR to create amplicons of varied sizes that are unique to several DNA sequences. Targeting many sequences at once allows for the possibility of obtaining more data from a single test run than would otherwise be possible it would take more time to accomplish as well as several times the amount of reagents. The amplicon sizes, or their base pair lengths, should be diverse enough to create discrete bands when seen by gel electrophoresis, and annealing temperatures for each of the primer sets must be adjusted to operate properly within a single reaction. In our study and by using multiplex PCR, we have identified 2 isolates from beef burger samples as *Salmonella enterica* ser. Enteritidis and one as *Salmonella enterica* ser. Typhimurium.One isolate recovered from kofta was identified as *Salmonella enterica* ser. Enteritidis and another one was *Salmonella enterica* ser. Dublin. An isolate from was found to be *Salmonella enterica* ser. Enteritidiswhile four isolates from chicken samples were identified as 1 *Salmonella enterica* ser. Typhi, 1 *Salmonella enterica* ser. Dublin and 2 *Salmonella enterica* ser. Gallinarum Pullorum.

CONCLUSION

In conclusion, fast diagnosis and epidemiological research for salmonellosis can both benefit greatly from the bacterial identification and screening of the *inv*A gene using PCR-based methods.*Salmonella enterica* ser.Enteritidis,*Salmonella enterica* ser.Typhimurium, *Salmonella enterica* ser. Typhi, *Salmonella enterica* ser. Dublin, and *Salmonella enterica* ser. Gallinarum-Pullorum have all been identified form different sample types using a multiplex-PCR method which was found quick and accurate.

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