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Multiplex PCR-based detection of *Salmonella* Typhimurium and *Salmonella* Enteritidis in Specific Pathogen Free (SPF) and Commercial Eggs

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Abstract

Salmonella serovars are one of the major bacterial causes of food borne diseases. Eggs are commonly identified as food sources responsible for salmonellosis outbreaks. This study aimed to isolate *Salmonella* Typhimurium and *Salmonella* Enteritidis from 1750 hens' eggs, and use of multiplex polymerase chain reaction (Multiplex PCR) in the identification of different *Salmonella* serovars from eggs. The incidence of salmonellae among the Balady eggs yolk was 1.3%, while the incidence was 1.2% among white and brown eggs samples (each). *S.* Typhimurium and *S.* Enteritidis were identified (0.6 and 0.5% respectively). The isolates were confirmed using *fliC*, *sefA* genes and gene specific for genus *Salmonella*. All albumen samples negative for isolation of isolation of salmonellae by culture method were retested by PCR.

From the retested albumen samples 3%, 8.4% and 6% collected from Balady, white and brown eggs respectively were positive for *Salmonella* serovars using Multiplex PCR. No salmonellae could be detected from specific pathogen free (SPF) eggs using both PCR and conventional methods.

Keywords: Eggs; SPF; Multiplex PCR; S. Typhimurium; S. Enteritidis

Introduction

Contamination of eggs has been identified as one of the major causes of food borne *Salmonella* [1]. In the United States all cases of *Salmonella* contamination of eggs were reported to the Centre for Disease Control and Prevention [2]. There are two pathways for eggs to become internally contaminated with *Salmonella*, direct contamination occurs during the formation of an egg in the ovary and oviduct of hens; whereas, indirect contamination occurs after penetration of salmonellae the egg shell membrane [3].

Salmonella Pullorum or *Salmonella* Gallinarum in the ovules before ovulation likely and probably constitutes the chief mode of vertical transmission [4]. The majority of human illnesses caused by *Salmonella* Enteritidis are attributed to the consumption of contaminated eggs [5].

The aim of this study was to determine *S.* Typhimurium and *S.* Enteritidis in eggs of Balady, white, brown and SPF layer breed collected from different governorates using conventional microbiology detection compared to that detected using Multiplex-PCR technique.

Materials and Methods

Samples

Total 1750 eggs were collected from Balady (n=1000), brown (n=250), white (n=250) and SPF (n=250) eggs from Kafr El sheikh, Elqalubia, El Monofia, Al Fayoum, EL Menia farms and from the SPF egg producing project (Koom Ousheem-Al Fayoum), Egypt. Egg yolk and egg albumins were collected from each egg and these samples were cultured within 24 hrs from collection.

Identification of Salmonellae

Under complete sterile condition each egg was cleaned by cotton swab soaked in alcohol. The egg was broken in a Petri dish plate then egg yolk and egg albumin were collected by two separate syringes. Detection of *Salmonella* was carried out according to ISO 6579: (2002) [6]. The samples were cultured on xylose lysine deoxycholate (XLD) and brilliant green agar (BGA) plates. The suspected colonies on XYD and BGA plates were picked up for microscopically examination by Gram's stain before being transferred into semisolid and slope agar for preservation and further identification. *Salmonella* isolates were identified biochemically and serologically as reported in previous literatures [7,8].

Detection of the genus *Salmonella* using the multiplex PCR-based assay

DNA was extracted from the examined samples using QIAamp® DNA Mini kit (catalog no. 51304, QIAGEN GmbH, Germany) according to manufactures recommendations. The PCR was conducted according to modified Oliveira protocol using specific primers as shown in table 1 [9]. The primers were prepared by Sigma Company in Germany according to Soumet et al., [10] and amplified PCR products were analyzed gel electrophoresis in 1% agarose gel.

Target sequence	Primer sets	Primer sequence 5'→3'	Amplification region (bp)
Random sequence	ST11	GCCAACCATTGCTAAATTGGCGCA	429
	ST15	GGTAGAAATTCCCAGCGGGTACTGG	
fliC gene	Fli15	CGGTGTTGCCCAGGTTGGTAAT	559
	Tym	ACTCTTGCTGGCGGTGCGACTT	
sefA gene	Sef167	AGGTTCAGGCAGCGGTTACT	312
	Sef478	GGGACATTTAGCGTTTCTTG	

Table 1: Primers used for the detection of *Salmonella* species [10].

Results

Detection of the *Salmonella* serovars among the examined eggs

The highest number of isolates was recovered from the Balady eggs collected from Kafr El sheik (2.7%) and EL Monofia (3%). The incidence was 1.2% from both the white and brown eggs as shown in table 2. All isolates of salmonellae were recovered from yolk samples only. The results revealed that 13 serovars (1.3%) were isolated from the yolk samples of Balady eggs and serotyped as 7 *S.* Typhimurium and 6 *S.* Enteritidis. Three serovars (1.2%) were isolated from the yolk of white eggs and serotyped as 2 *S.* Typhimurium and one *S.* Enteritidis. Also 3 serovars (1.2%) were isolated from the yolk of brown eggs and serotyped as 2 *S.* Enteritidis and one *S.* Typhimurium as shown in table 3. It is also clear that all SPF eggs were free from salmonellae infections.

Type of eggs	Number of the examined eggs	Number of *Salmonella* isolates	Percentage (%)	Governorates
White	250	3	1.20%	
	150	2	1.30%	Kafr El sheik
	100	1	1%	Elqalubia
Brown	250	3	1.20%	
	100	1	1%	Elqalubia
	150	2	1.30%	El Monofia
Balady	1000	13	1.30%	
	250	2	0.80%	EL Sharkia
	250	2	0.80%	Al Fayoum
	250	2	0.80%	EL Menia
	150	4	2.70%	Kafr EL Sheik
	100	3	3%	EL Monofia
SPF	250	-	0%	Al Fayoum
Total	1750	19	1.09%	

Table 2: Prevalence of *Salmonella* serovars recovered from the examined eggs.

Confirmation of the isolates using multiplex PCR

Using primers specific for Genus *Salmonella*, for *S.* Enteritidis (*sefA* gene) and *S.* Typhimurium (filC gene) serovars, Multiplex PCR was used to identify the specific isolates. All isolates were positive for amplification of 429 bp specific for Genus *Salmonella*. Furthermore, *S.* Enteritidis and *S.* Typhimurium isolates were positive for amplification of 312 bp and 559 bp respectively (Figure 1).

Type of eggs	*Salmonella* serovars				Total	
	S. Enteritidis		*S.* Typhimurium			
	No.	%	No.	%	No.	%
Balady eggs(1000)	6	0.6	7	0.7	13	1.3
White eggs(250)	1	0.4	2	0.8	3	1.2
Brown eggs (250)	2	0.8	1	0.4	3	1.2
SPF eggs (250)	-	0	-	0	-	0
Total 1750	9	0.5	10	0.6	19	1.3

Table 3: *Salmonella* serovars isolated from the egg yolk of the Balady, brown and white eggs.

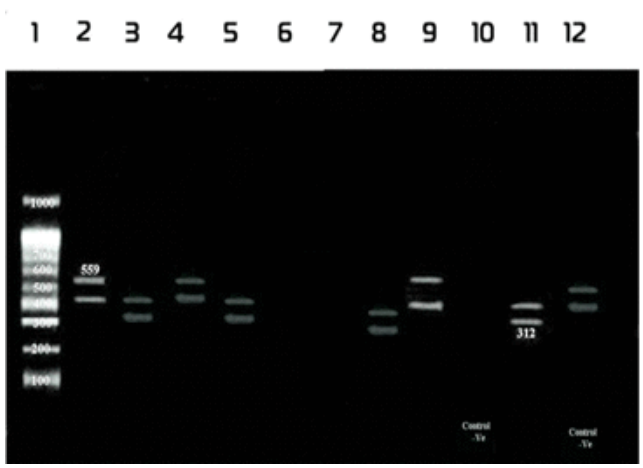

Figure 1: Agarose gel electrophoreses showing amplification of 429, 559 and 312 bp fragments from the extracted DNA of *Salmonella* isolates. Lane 1: 100 bp DNA marker (GibcoBRL), Lanes 2, 4, 9 and 12: positive amplification of 559 bp fragment of *S.* Typhimurium isolates, Lanes 3, 5, 8 and 11: positive amplification of 312 bp fragments of S. Enteritidis isolates, Lane 12: positive control (S. Typhimurium ATCC 13076), Lanes 6 and 7 negative control (*S. aureus* ATCC 29737) and Lane10: negative control (distilled water).

Direct detection of the *Salmonella* from egg albumin using the multiplex PCR

All albumen samples collected from the examined eggs were retested by m PCR for detection of *S.* Enteritidis and *S.* Typhimurium. It is clear that 66 (4.4%) out of 1500 albumin samples were positive for salmonellae, 48 (3.2%) and 18 (1.2%) samples were positive for *S.* Enteritidis and *S.* Typhimurium respectively. Among the Balady eggs, 21 (2.1%) and 9 (0.9%) samples were positive for *S.* Enteritidis and *S.* Typhimurium respectively. From the white egg albumin samples, 17 (6.8%) and 4 (1.6%) samples were *S.* Enteritidis and *S.* Typhimurium respectively. The albumin samples of the brown eggs recorded 10 (4%) and 5 (2%) positive samples for *S.* Enteritidis and *S.* Typhimurium respectively as shown in table 4 and figure 2.

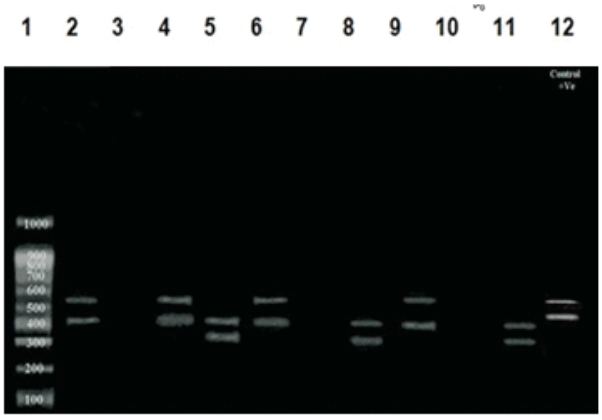

Figure 2: Agarose gel electrophoreses showing amplification of 429 bp, 559 bp and 312 bp fragments from the egg albumen samples. Lane 1: 100bp DNA marker (GibcoBRL), Lanes 2, 4, 5, 6, 8, 9,11 and 12: positive amplification of 429 bp fragment of *Salmonella* species, Lanes 2, 4, 6, 9 and 12: positive amplification of 559 bp fragment of *S.* Typhimurium, Lanes 5, 8 and 11: positive amplification of 312 bp fragment of *S.* Enteritidis, Lane 12: positive control *S.* Typhimurium (ATCC 13076), Lane 10: negative control *S. aureus* (ATCC 29737), Lanes 3 and 7: negative control distilled water.

Types of egg	Number of examined albumin samples	Amplified PCR product		
		429 bp (Genus *Salmonella*) n (%)	312 bp (*S.* Enteritidis) n (%)	559 bp (*S.* Typhimurium) n (%)
Balady	1000	30 (3%)	21 (2.1%)	9 (0.9%)
White	250	21 (8.4%)	17 (6.8%)	4 (1.6%)
Brown	250	15 (6%)	10 (4%)	5 (2%)
Total	1500	66 (4.4%)	48 (3.2%)	18 (1.2%)

Table 4: Direct detection of the *Salmonella* from egg albumin samples using the multiplex PCR.

Discussion

Salmonella contamination of eggs has been identified as a public health concern worldwide. Globally, *Salmonella* is one of the most prevalent causes of food borne illness [3].

The present data revealed that 19 *Salmonella* isolates were isolated from 1500 examined Balady, white and brown eggs (1.3%). Earlier *Salmonella* was isolated by Jones et al., [11] from 72.0% of all samples collected from the laying house environment (flush water, ventilation fan, egg belt, and egg collector samples).

It is clear that 10 *S.* Typhimurium and 9 *S.* Enteritidis were identified serologically with incidence of 0.6 and 0.5% respectively. *Salmonella* Enteritidis and *S.* Typhimurium as well as other serotypes have been isolated from egg shells and egg content [12].

The two most commonly identified causative agents of food borne salmonellosis are *Salmonella enterica* serotypes Typhimurium and Enteritidis [13]. Both serotypes have the ability to colonize the reproductive organs of hens and are major causes of food borne illness [3].

S. Enteritidis is more commonly linked to contaminated eggs, except in Australia, where the majority of egg-related food borne salmonellosis is caused by *S.* Typhimurium [14-16]. It has been concluded that *S.* Enteritidis could penetrate the egg shell easier than other serotypes so they supposed that horizontal transmission of *Salmonella* in eggs is of less importance than the vertical transmission [17]. Hen's eggs are the most important vehicle of the *S.* Enteritidis infection in humans [18].

The most commonly used technique for *Salmonella* detection is the conventional culture technique. The polymerase chain reaction (PCR) method required only 2 days, compared to the 5 days required by conventional selective enrichment and serological tests for *Salmonella* serovars the culture method and the sensitivity of this assay was approximately less than 1 CFU/600 g of egg pool [19].

A polymerase chain reaction for the specific detection of the gene sequence, *sefA*, encoded by all isolates of *Salmonella* Enteritidis, was developed previously by Woodward and Kirwan [20].

The PCR assay proved by Seo et al., [19] to be a rapid and highly sensitive test for detection of low concentrations of *Salmonella* in egg samples. PCR represents a rapid procedure to detect *Salmonella* in a food sampled. In this study, *sefA* and *filC* genes were amplified to confirm the isolates as well as to detected *S.* Enteritidis and *S.* Typhimurium directly from egg albumen samples. While no albumen sample was detected by the microbiological method, 21 (2.1%) and 9 (0.9%) samples from Balady eggs, 17 (6.8%) and 4 (1.6%) samples from white egg albumin samples and 10 (4%) and 5 (2%) from brown eggs were positive for *S.* Enteritidis and *S.* Typhimurium respectively. PCR is a sensitive method with a superior ability to detect *Salmonella* spp. in the presence of other competing bacteria [21,22].

All examined albumen samples were negative for isolation of salmonellae by culture method. Egg white proteins, such as lysozyme and ovotransferrin, are well known to play important roles in defense against bacterial invader Baron et al. [23]. Using PCR, 3%, 8.4% and 6% albumen samples collected from Balady, white and brown eggs respectively were positive for *Salmonella* serovars. *Salmonella* DNA could be detected from infertile eggs which incidence was higher than that by bacteria isolation [24]. *Salmonella* strains grow better in fresh egg white than in egg white of 2 or 3 weeks old [17]. In fresh eggs, only few *salmonella*s are present and as albumen is an iron-restricted environment, growth will only occurs once storage-related changes to vitelline membrane permeability, which allows salmonellae to invade yolk contents, have taken place, when this happens high populations are achieved in both yolk contents and albumen [25]. In the present study 48 and 18 cases were positive to *S.* Enteritidis and *S.* Typhimurium respectively using m PCR. Baron et al., [23] reviewed critically assesses the available evidence on the antimicrobial components of egg white. In addition, mechanisms employed by *S.* Enteritidis to resist egg white exposure are also considered along with various genetic studies that have shed light upon egg white resistance systems. The egg-contamination capacity of *S.* Enteritidis includes its exceptional survival capability within the harsh conditions provided by egg white [23].

Multiplex PCR is a sensitive method with a superior ability to detect *Salmonella* spp. in the presence of other competing bacteria. Although *Salmonella* contamination of eggs is a complex issue that is influenced by many variables, making it difficult to implement appropriate management strategies. Further research is required to explore different protocols to ensure control of *Salmonella* through temperature and pH of food products. There is also a need to re-educate food handlers and consumers of the risk from raw eggs and cross contamination of food products and reduce the public health risk.

Conflict of Interest

The authors declare that there is no conflict of interests.

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