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Identification of the *Salmonella* Genes *Yafd* and *Xtha*: A Step into the Control of *Salmonella* Food Poisoning Associated with Consumption of Eggs in Egypt

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Abstract: All serotypes of non-typhoid *Salmonella* are considered as a potential hazard to human health and measures to prevent food-borne salmonellosis should therefore be directed there wise. Here we sought to report the identification and incidence of *S. enteritidis* and *S. typhimurium yafD* and *xth*A gene families that are responsible for their persistence in egg albumen and their survival in egg are important for their transmission to the human population. The total incidence of *Salmonella* in the albumen samples was detected as 10.1% of the samples [*Salmonella enteritidis* (7.1%) and *S. typhimurium* (3.1%)]. As carrier for the *yafD* (86.7%) and *xthA* (13.3%) genes, *S. enteritidis* accounted for 85.0% of the *Salmonella* serotype isolates. On the other hand, *S. typhimurium* carrying the *xthA* gene, accounted for 100% of the total egg albumen *Salmonella* serotypes with the absence of the *yafD* gene from all of the *S. typhimurium* isolates.

Key words: *S. enteritidis* · *S. typhimurium* · Resistant Genes · Egg Albumen

INTRODUCTION

Foodborne salmonellae are estimated to cause ≈1.3 million illnesses, 15,000 hospitalizations and 500 deaths and cause severe economic losses which approach from $0.5 to 2.3 billion per year in the United States and throughout the world [1]. Most reports have mentioned *S. enterica* serovar typhimurium and *S. enterica* serovar Enteritidis as the most common causes of human salmonellosis worldwide [2-7].

Several epidemiologic studies carried out have indicated the important role of eggs as a major vehicle in the transmission of the organisms to human consumers through consumption of raw or partially cooked eggs [8, 9]. In Egypt, consumption of poultry products has risen and *S. enteritidis* infections in poultry have increased in recent years in Egypt having a significant economic impact on the poultry industry, especially the egg industry. The incidence of *S. enteritidis* and *S. typhimurium* in eggs has reached 16.7 and 13.3%, respectively [10].

Several authors have shown that egg albumen (a hostile and stressful environment) can control the proliferation of *S. enteritidis* and to be bactericidal toward *Salmonella* [3, 11-13]. The albumen induces the expression of a number of genes, whose products are essential for *Salmonella* to repair cell damage to overcome host defense mechanisms and may therefore be called true virulence determinants [14]. Such true virulence determinants were recognized by Lu *et al.* [12] and Clavijo *et al.* [15] who reported that genes *yafD* and *xthA* are necessary for *S. enteritidis* to survive in egg albumen, an environment that is generally hostile to bacteria [9].

In the present study, the primary objective of this investigation was to determine the *xth*A and *yafD* genes responsible for the survival of *S. enteritidis* and *S. typhimurium* in chicken egg by identifying these genes.

MATERIALS AND METHODS

Sample Collection: Overall 750 chicken eggs 60 g ± 3 g by weight, freshly layed, were collected from local farms, open markets and supermarkets located in Cairo during the period of June to August 2010. The sampling locations were chosen according to their distance from the laboratory. All samples were evaluated by the quality

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of egg shell, by ovoscopy, as with no defects and within the recommended period for consumption. The samples were processed within 2-3 hours of purchase.

Bacterial Isolation: Chicken eggs from local farms, open markets and supermarkets were disinfected by immersion in 70% ethanol for 30 minutes and then dried, scorched and cracked into a sterile container. After aseptic breaking, the albumen was separated and diluted in buffered peptone water (decimal dilution). Egg albumen was beaten with an electric mixer for 3 min at the lowest speed. Bacteriological samples were analyzed by the ISO 6579 European International Standard Method [16]. Briefly, one milliliter from each beaten egg albumen samples was pre-enriched in 9 ml buffered peptone water (BPW) supplemented with 35 mg/L of ferric sulfate (Fisher Scientific) (BPW; Becton, Dickinson and Co. Oakville, Ontario, Canada) at a ratio of 1:10. After incubation at 37°C for 18 h, 1 ml of BPW was transferred to 9 ml of Muller-Kauffmann tetrathionate novobiocin broth (MKTTn broth; Oxoid, United Kingdom) as a selective enrichment and incubated at 37°C for 24 h. One milliliter of the MKTTn broth was then spread on xylose lysine desoxycholate agar, brilliant green agar and Hektoen enteric agar (Oxoid) and incubated at 37°C for 24 h. Presumptive *Salmonella* colonies were characterized by biochemical assays [17] and serotyped using Kauffmann-White typing scheme [18]. One milliliter of BPW which had been incubated at 37°C was saved for the 37°C Rappaport-Vassiliadis (RVs) selective enrichment broth for the PCR-RVs test.

DNA Extraction and Purification: Following the protocol of Oliveira *et al.* [19], bacterial cells were pelleted from 1 ml of RVs broth by centrifugation (2000 × *g* for 4 min at room temperature) and the supernatant discarded. DNA was extracted by re-suspending the pelleted cells in 444 µl of TE (10 mmol l^{-1} Tris-HCl pH 8.0, 1 mmol l^{-1} EDTA) containing 30 µl lysozyme (50 mg ml^{-1}) (Pharmacia Biotech, Piscataway, NJ, USA) and incubating the suspension at 4°C for 30 min, after which 25 µl of 10% sodium dodecyle sulphate (SDS) and 1.25 µl of proteinase K (20 mg ml^{-1}; GibcoBRL, Paisley, UK) were added and the solution was incubated at 55°C for 30 min. For DNA purification, 500 µml of 1:1 phenol: chloroform (pH 8.0) was added and the mixture agitated for 5-10 s and then centrifuged (16 000 × *g* for 4 min at room temperature), after which the supernatant was extracted once more with 1: 1 phenol: chloroform and then with chloroform only. The purified DNA was precipitated by adding 35 µl of 3 mol l^{-1} sodium acetate (pH 7.0) and 350 µl of cooled isopropanol and incubating the mixture at -20°C for 30 min, after which the DNA was pelleted by centrifugation (16 000 × *g* for 10 min at 4°C) and the supernatant discarded, the pelleted DNA being washed with 1 ml of 80% cooled ethanol and re-suspended in 50 µl of TE for immediate use or storage at -20°C.

PCR Screening for The *Sefa* And *Flic* Genes for Identification of *S. enteritidis* and *S. typhimurium*: The multiplex PCR primer sets (Table 1) previously assessed as specific for *S. enteritidis* (Sef167-Sef478) and *S. typhimurium* (Fli15-Tym) were used, together with ST11-ST15 primers specific for the genus Salmonella [20].

DNA Amplification: PCR amplifications were carried out in a GeneAmp PCR System 9600 Thermocycler (Perkin-Elmer Instruments, Norwark, CT, USA) using conditions adopted by Soumet *et al.* [20]. All reactions were carried out in a final volume of 50 µl in micro-amplification tubes (PCR tubes). The reaction mixture consisted of 5 µl (200 ng) of the extracted DNA template added to the PCR tube containing 5 µl 10X PCR buffer (75 mM Tris- HCl, pH 9.0, 2 mM $MgCl_2$, 50 mM KCl, 20 mM $(NH4)_2SO_4$), 1 µl dNTPs (40 µM), 1 µl (1U Ampli Taq DNA polymerase), 0.5 µl (50 pmol) from the forward and reverse primers (Table I) of each of the three primers. The volume of the reaction mixture was completed to 50 µl using Deuterium Depleted Water (DDW). The vial was overlayed with 40 µl paraffin oil. PCR cycling conditions were adjusted as follows: Initial denaturation at 94°C for 5 min, followed by 35 cycles of denaturation at 94°C for 1 s, annealing at 56°C for 1.5 min and extension at 72°C for 30 s, with a final

Table 1: List of primers used in the PCR-based assay for the detection of *Salmonella* species.

Target sequence	Primer sets	Length	Primer sequence 5' → 3'	Amplification region (bp)
Random sequence	ST11	24	GCCAACCATTGCTAAATTGGCGCA	429
	ST15	24	GGTAGAAATTCCCAGCGGGTACTGG	
FliC gene	Fli15	22	CGGTGTTGCCCAGGTTGGTAAT	559
	Tym	22	ACTCTTGCTGGCGGTGCGACTT	
SefA gene	Sef1 67	20	AGGTTCAGGCAGCGGTTACT	312
	Sef478	20	GGGACATTTAGCGTTTCTTG	

extension at 72°C for 10 min. Amplification products were separated by electrophoreses on 1.2% agarose gel containing 5 µg ml^{-1} ethidium bromide with a 100-bp ladder (GibcoBRL) as molecular weight marker.

PCR Screening for the *Salmonella* Genes *yafd* and *xthA*: Two independent single PCR reactions, each with one set of primers [15], were made for each DNA template.

Gene="*yafD*" (1800 bp)/
F 5' AACTGAGAATTCGTGTAGGAGCTGCTTC 3'
R 5' CCACATGAATTCCATATGAATATCCTCCTTAG 3'
Gene="*xthA*" (226 bp)/
F 5' AGTCCACGACGAAATGTTCC 3'
R 5' CGGGAAATAGCCGTTAATGA 3'

Each reaction mixture was carried out in a final volume of 50 µl with one set of primers (1 µl [50 pmol]) from the forward and reverse primers and DNA template (1 µl [200 ng]) extracted from the RVs broth. All other PCR reaction components remained as described above. The thermal cycler was adjusted as follows: initial denaturation at 94°C for 5 min, followed by 35 cycles of denaturation at 94°C for 1 s, annealing at 55°C for 1 s and extension at 72°C for 21 s, with a final extension at 72°C for 7 min. Amplification products were separated and observed as previously described.

RESULTS

The egg albumen were individually cultured by incubating them for 72 h at room temperature and then testing them for the presence of *S. enteritidis* and *S. typhimurium* by plate culture were culture negative.

In the multiplex PCR, four distinct results were obtained: for *S. typhimurium*, two amplified products of 429 and 559 bp; for *S. enteritidis*, 429 and 312 bp amplified products; one amplified product of 429 bp was noted for all serotypes of Salmonella; and no amplified product for bacterial strains related to Salmonella (Fig. 1).

The total incidence of *Salmonella* in the albumen samples was detected as 10.1% (76/750) of the samples. *Salmonella enteritidis* was recovered from 53/750 samples (7.1%) and *S. typhimurium* from 23/750 samples (3 %).

The Single PCR produced an intense band of the expected 1800 bp targeting the *yafD* gene (Fig. 2) and the 226 bp targeting the *xthA* gene (Fig. 3) with all the *Salmonella* strains.

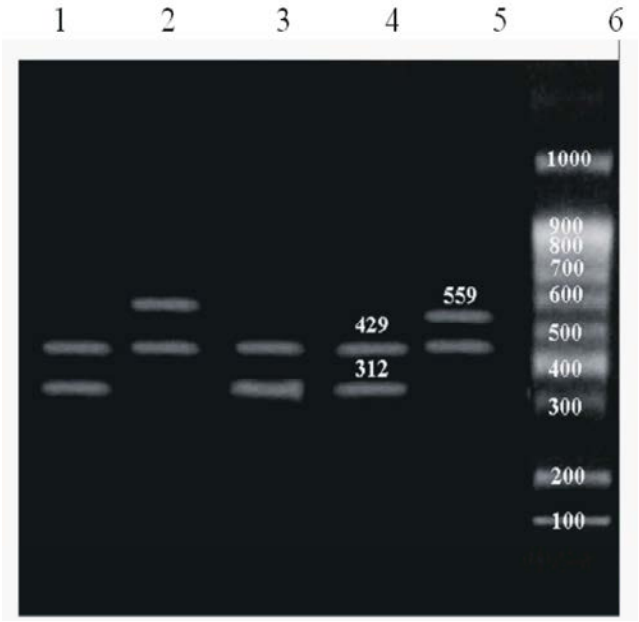

Fig. 1: Detection of *Salmonella* serovars in egg albumen by m-PCR.
Lane 1: Positive control of *S.* Enteritidis (ATCC 13076), Lanes 1-5: Positive amplification of 429 bp fragment (random sequence) of *Salmonella* species, Lanes 2 and 5: Positive amplification of 559 bp fragment (*fliC* gene) of *S. typhimurium*, Lanes 3 and 4: Positive amplification of 312 bp fragment (*sefA* gene) of *S.* Enteritidis and Lane 6: 100bp marker.

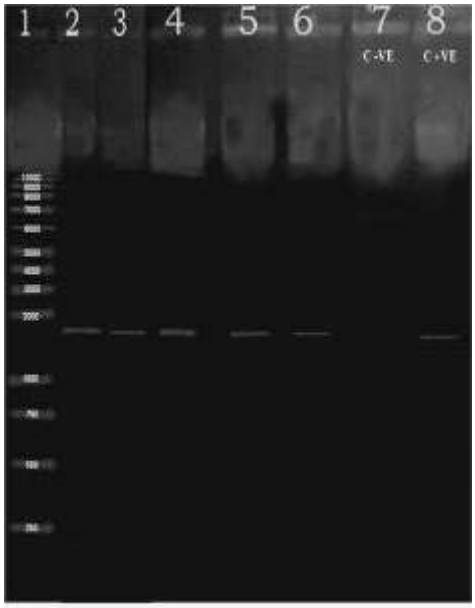

Fig. 2: PCR sensitivity and detection test of the resistant gene *yafD* (1800 bp) in the egg albumen.

Lane 1: 250 bp marker, Lanes 2-6: Positive amplification of the resistant gene *yafD* on *S*. Enteritidis field strains, Lane 7: Negative control *S. aureus* (ATCC 29737) and Lane 8: Positive amplification of the *yafD* gene on a *S*. Enteritidis (ATCC 13076) standard strain.

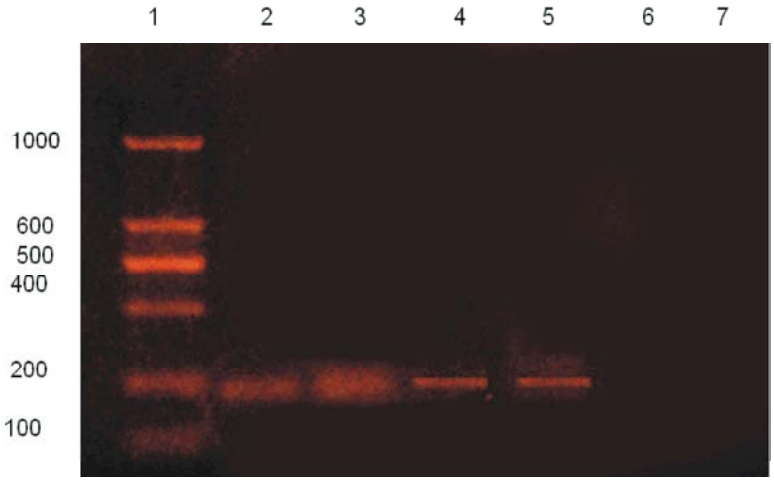

Fig. 3: PCR sensitivity and detection test of the gene *xthA* (226 bp) in the egg albumen.

Lane 1: 100 bp marker, Lane 2: Positive amplification of the *xthA* gene on a *S. enteritidis* (ATCC 13076) standard strain, Lanes 3-5: Positive amplification of the *xthA* gene on a *S. typhimurium* field strain, Lane 6: Negative amplification of the *xthA* gene on a *S. typhimurium* field strain and Lane 7: Negative control S. *aureus* (ATCC 29737).

Table 2: Detection of the *yafD* and *xthA* genes in *S. enteritidis* and *S. typhimurium* by the use of specific primers.

	S. enteritidis				*S. typhimurium*			
Poultry egg albumen samples	Positive for *S. enteritidis*	Positive for *S. enteritidis*[a] (carrying the *yafD* or/and *xthA* genes)	*yafD* gene[b]	*xthA* gene[b]	Positive for *S. typhimurium*	Positive for *S. typhimurium*[a] (carrying the *yafD* or/and *xthA* genes)	*yafD* gene[b]	*xthA* gene[b]
n= 750	53/750 (7.1%)	45/53 (85.0%)	39/53 (86.7%)	6/53 (13.3%)	23/750 (3 %)	17/23 (73.9%)	0/23 (0%)	17/23 (100%)

[a]Percentage calculated against the number of positive egg albumen samples to *S. enteritidis* and *S. typhimurium*

[b]Percentage calculated against the number of positive egg albumen samples to *S. enteritidis* and *S. typhimurium*

As carrier for the *yafD* (86.7%) and *xthA* (13.3%) genes, *S. enteritidis* accounted for 85.0% of the *Salmonella* species isolates (Table 2). On the other hand, *S. typhimurium* carrying the *xthA* gene, accounted for 100% of the total egg albumen *Salmonella* species isolates (Table 2) with the absence of the *yafD* gene from all of the *S. typhimurium* isolates.

DISCUSSION

There have been numerous alerts concerning *Salmonella* contaminated meat, meat products and poultry notified through the rapid alert system for food and feed [21]. Taking these needs into account, FAO and WHO initiated work on the pathogen-commodity combinations identified as priority of which *S. enteritidis* in eggs as one of the issues, especially after an apparently new virulent strain of *S. enteritidis* appeared in the 1980s, with the ability to infect the eggs of poultry resulting in a pandemic spread in humans, with contaminated eggs and egg products being the principal vehicle.

S. enteritidis can contaminate eggs through transovarian transmission during egg development in the infected chickens [9] by being deposited into both albumen and yolk. It is more frequently deposited into the albumen, especially in naturally contaminated eggs [9, 22].

Bacteriological methods express the actual infection status of the eggs by isolation of the actual infectious agent/s. Conventional cultural methods for detecting *S.* enteritidis in egg samples are generally laborious and time consuming. However, the FDA's Bacteriological Analytical Manual (BAM) specifies preenrichment, selective enrichment and selective plating for the recovery of *Salmonella* spp. in foods to allow for the recovery of injured cells and detection of low numbers of cells [23]. The pre-enrichment method for detection of *S. enteritidis* in eggs was superior to the direct plating method [23]. In addition, recovery of *Salmonella* from albumen has proven most difficult because of the presence of innate iron binding and other antimicrobials in egg white [24]. The low contamination rate and the antimicrobial effect of egg albumen have contributed to the difficulty in detecting *S. enteritidis* in raw eggs [23, 25]. On those bases, the culture negative egg albumen recorded in the present investigation was not surprising as the use of standard microbiological techniques (SMT) was not effective in isolating *Salmonella* species from egg albumen. The recorded data showed that PCR with selective enrichment detected more positive samples than the SMT from egg albumen.

Consequently, a control programme needs a supporting monitoring programme using molecular methods to obtain a true picture of *Salmonella* status. PCR improves both the speed of detection and the level of sensitivity at which organisms can be detected [19]. Therefore, the on-farm microbiologic testing and control procedures to reduce *S. enteritidis* and *S. typhimurium* contamination of eggs should be supplemented by the strategic use of genetic methods to ensure that emerging serovars are also detected. In a previous study, Oliveira *et al.* [19] found that, a PCR assay combined with Rappaport-Vassiliadis (RVs) selective enrichment broth (PCR-RVs) was more sensitive and efficient than the PCR assay combined with NS enrichment broth (PCR-NS) and standard microbiological techniques (SMT) with respect to their ability to detect *Salmonella* sp. and identify *S. enteritidis* and *S. typhimurium*. However, it still requires double enrichment steps to achieve high sensitivity equivalent to conventional culture methods [23]. Thus, the BPW and PCR-RVs steps were included in our study.

Stress-induced survival mechanisms enable the serotype *S. enteritidis* to persistently cope with the antimicrobial compounds and thus survive in egg albumen [15,26]. Lu *et al.* [12] proposed that *YafD* provids a survival advantage to *S. enteritidis* in eggs by repairing DNA damage caused by egg albumen and that it may be one of the biologic determinants that contribute to the epidemiological association of *S. enteritidis* with egg products. The differential survival of *S. enteritidis* and *S. typhimurium* in egg albumen [15] may contribute to the epidemiological differences regarding the food vehicles through which they are transmitted. In the present investigation, *S. enteritidis* had a unique set of egg-resistant genes (*yafD* and *xthA*) while *S. typhimurium* carried only one gene (*xthA*). This diversity in genes present in both *S. enteritidis* and *S. typhimurium* result in significant differences in their functions in egg albumen resistance. The differential regulation of genes shared between *S. enteritidis* and *S. typhimurium* lead to the increased resistance of *S. enteritidis* [15].

In the present investigation, *S. enteritidis* carrying the *yafD* and *xthA* genes represent 85% of the total *S. enteritidis* isolated and *S. typhimurium* carrying the *xthA* gene represent 73.9% of the total *S. typhimurium* isolated. Clavijo *et al.* [15] identified genes that play significant roles in the survival of *S. enteritidis* and *S. typhimurium* in egg albumen. These genes might represent significant factors that had led *S. enteritidis* and *S. typhimurium* to become a major food-borne pathogen over the past few decades.

Foodborne illness, related to microbiological hazards in foods [27] is among the most widespread public health problems and creates social and economic burdens as well as human suffering, making it a concern that all countries need to address. Many countries have trade restrictions for *Salmonella* and trade between countries has often been interrupted by *Salmonella*-contaminated consignments [28]. Yet, there is no international standard and there is no legal basis for not trading eggs with *Salmonella*. Therefore, Microbiological Risk Assessment (MRA) must be used to justify the introduction of more stringent standards for imported foods. An important prerequisite for MRA is the need to develop the capacity for microbiological analysis in order to improve the hazard characterization. *Salmonella* genes assessments provide information that may be useful in determining the impact that intervention strategies have on reducing cases of salmonellosis from contaminated eggs. This information is of particular interest to the Codex Alimentarius in their work on the elaboration of standards, guidelines and related texts for the management of risks posed by microbiological hazards in foods.

Any serotype, including those that infect animals or colonize their intestine, must be considered as a potential hazard to human health and measures to prevent food-borne salmonellosis must therefore be directed at all serotypes of *Salmonella* [30, 31]. The implementation of such a strategy requires a supporting surveillance programme to detect the prevalence of zoonotic serotypes carrying the *yafD* and *xthA* genes and prevent their build-up in the production chain. If no interventions are made at this early stage, these serotypes and others, could later spread widely, perhaps reaching epidemic proportions. Although further control of *S. enteritidis* and *S. typhimurium* requires limiting the spread of *S. enteritidis* and *S. typhimurium* on farms, yet, the evolving of an oversight into the Egg Quality Assurance Program (EQAP) in Egypt must be initiated.

It can be concluded that, *Salmonella* infection in human can be reduced through upgrading methods of detection. Inclusion of the *Salmonella* genes *yafD* and *xthA* detection in the EQAP is a must.

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