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MOLECULAR CHARACTERIZATION OF *SALMONELLA* SPECIES ISOLATED FROM CHICKEN TABLE EGG CONTENT

SABER, A.S. [1] and ABEER H.A. [2]
[1] Animal Health Research Institute, Damanhour Branch, Egypt
[2] Animal Health Research Institute, Alexandria Branch, Egypt

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ABSTRACT

Salmonellosis is a foodborne illness caused by consumption of contaminated food. Infected raw or undercooked eggs are one of the major causes of salmonellosis, which lead to many public health problems. The aim of this study is isolation and identification of *Salmonella* spp. from the egg content samples, in addition, detection of some virulence gene (*avr*A, *inv*A and *stn*). Four hundred edible farm and balady eggs (200 of each) were randomly collected from different supermarkets and farms in El-Behera Governorate, Egypt. The obtained results revealed that the incidence of *Salmonella* species were 1 and 3% in examined farm and balady eggs, respectively and also 1% in balady eggs while couldn't be detected in farm eggs by direct molecular target genes detection. Serotyping of *Salmonella* isolates revealed that two different *Salmonella* serotypes were identified as; *S*. enteritidis (1strain), *S*. typhimurium (1 strain) from farm eggs and *S*. enteritidis (5 strains) and *S*. typhimurium (1 strain) from balady eggs. Six isolates of *Salmonella* serovars were molecularly identified, all had *avr*A gene at 422 bp, *inv*A gene at 284 bp and *stn* gene at 617 bp. In addition direct detection of virulence gene from egg content of ten egg samples for *Salmonella* revealed that 2 samples from balady eggs contain all target virulence genes (*avr*A, *inv*A *and stn*) with amplified products 422, 284 and 617 bp, respectively. Presence of *inv*A, *avr*A and *stn* gene in all isolates indicated that they are capable of producing gastroenteric illness to humans.

Key Words: *S*. enteritidis, *S*. typhimurium, edible eggs, virulence genes, PCR.

INTRODUCTION

Egg is an ideal source of nutrients for proliferation of both spoilage and pathogenic contaminating microorganisms. Fresh egg has three structures, which are an outer waxy shell membrane, the shell and the inner shell membrane and each is effective to some degree of retarding the entry of microorganisms (Jay *et al*., 2005).

Consumers prefer the egg in which the albumen is firm, the yolk has a dense color, the egg is of an appropriate size with intact shell, and is free from pathogens (Samiullah and Chousalkar, 2014).

Salmonella is a genus of rod-shaped (bacillus) Gram-negative bacteria of family Enterobacteriaceae. The two species of *Salmonella* are *Salmonella* enterica and *Salmonella* bongori *S*. enterica is the type species and is further divided into six subspecies that include over 2,600 serotypes (Gal-Mor *et al.,* 2014).

Corresponding author: Dr. SABER, A.S.
E-mail address: sabersaad284@yahoo.com
Present address: Animal Health Research Institute, Damanhour Branch, Egypt

Eggs are contaminated with *Salmonella* by two routes; vertical transmission by transport *Salmonella* from infected reproductive tissues to egg prior to shell formation and horizontal transmission by contamination of egg shell with animal feces and through environmental vectors such as farmers, pets and rodents. Improperly storage and shell damage may facilitate contamination with *Salmonella* (Poppe *et al*., 1998).

Salmonellae considered as the most common bacteria-contaminating egg shell (Ricke *et al.,* 2001). *Salmonella* enteritidis and *Salmonella* typhimurium are the most frequent *Salmonella* serotypes found inside the eggs that caused food poisoning. In addition, the organism causes human salmonellosis, which may range from gastroenteritis to typhoid (Tan *et al*., 2012).

Salmonella enteritidis is considered an emerging predominant serotype isolated from poultry eggs (Huehn *et al*., 2010). *Salmonella* enterica serovar enteritidis have the capability to infect developing eggs within the oviduct, and therefore contaminated eggs act as an ecological amplifier (Gast *et al.,* 2011).

Complex pathogenesis is characteristic of *Salmonella* infection. The virulence of *Salmonella* is encoded by multiple genes, which are clustered on *Salmonella* pathogenicity islands (SPIs). SPIs have the potential to contribute to the overall pathogenesis of the bacterium (Hensel, 2004). Genomic variability among bacterial strains arises primarily because of horizontal gene transfer (Suez *et al*., 2013). This inherent variability is likely the source of the various pathogenicities among non-typhoidal *Salmonella* strains. Consequently, characterization of the virulence gene repertoire by PCR has been used to profile the virulence of *Salmonella* (Shah *et al.,* 2011).

Salmonella–specific PCR test in conjunction with traditional isolation methods could be effective in providing a more accurate profile of the prevalence of *Salmonella* in poultry products such as eggs (Ahmed *et al.,* 2014). PCR method can be used as an important technique in the diagnosis of virulence genes (*inv*A and *stn*) of *Salmonella* serotypes. In addition to the importance of *inv*A gene that could be used as a marker for rapid and accurate detection of *Salmonella* species (Fekry *et al.,* 2018)

The standard conventional cultural techniques to identify *Salmonella* spp. are time-consuming and can require up to 5 days for confirmation. Polymerase chain reaction (PCR) based methods combine simplicity with a potential for high specificity and sensitivity in detection of *Salmonella*. Amplification of *inv*A gene of *Salmonella* has been reported as a suitable target for PCR amplification, with potential diagnostic applications (Malorny *et al.,* 2003).

Pathogenic processes in salmonellosis are dictated by an array of factors that act in tandem and ultimately manifest in the typical symptoms of salmonellosis. Virulence genes encode products that assist the organisms in expressing its virulence in the host cells. Nucleic acid based techniques are being employed for the detection of various gene-encoded virulence factors *inv*A and *avr*A genes that associated with *Salmonella* pathogenicity islands (SPIs), and *stn* involved in enterotoxin production (Muthu *et al.,* 2014). Studies indicated that *Salmonella* species, which putatively possess virulence genes such as *hil*A and *inv*A, are consistently associated with severe illness compared with those, which lack such genes (Castagna *et al.,* 2005).

Because of the continuous consumer demands worldwide for eggs, periodical assessment is required to offer safe and good quality eggs for consumption, Therefore, this study was designed to determine the incidence of *Salmonella* species in egg content of both farm and balady eggs, In addition, investigation the presence of virulence genes (*avr*A, *inv*A and *stn*) in the isolated *Salmonella* species.

MATERIALS AND METHODS

1. Collection of samples:

Four hundred samples of edible eggs (200 farm eggs and 200 balady eggs) were collected randomly from different supermarkets and farms in El-Behera Governorate. Eggs were collected in sterile polyethylene bags and transferred in refrigerated containers to the laboratory for bacteriological investigation according to (Moosavy *et al*., 2015).

2. Bacteriological examination:

2.1. Preparation of egg content for culturing according to (Zubair *et al*., 2017)

The egg surface was sterilized by immersion in 70% alcohol for 2 min, air dried for 10 minutes and then cracked with a sterile knife. Egg contents were pipetted and poured into a sterile jar and homogenized.

2.2. Isolation and identification of *Salmonella* species (ISO, 2002)

For pre-enrichment, 1:10 dilution in buffered peptone water was prepared from homogenized egg content at ambient temperature and incubated for 18±2 hours for 37^{o}C±1^{o}C. Selective enrichment was applied by addition of 0.1 ml of culture to 10 ml of Rappaport vassiliadis (RV) broth and incubated for 24 hours at 41.5^{o}C±1^{o}C. Then a loopful of RV culture was streaked onto Xylose Lysine Deoxycholate agar (XLD) plates, plus Brilliant Green agar and incubated at 37°C for 24 hours. The pure red colonies with black centers suspected *Salmonella* were subjected to biochemical screening such as; sugar fermentation and H_2S production on triple sugar iron agar, decarboxylation of lysine, β-galactosidase reactions, indole production in tryptone broth and urea splitting ability in Christensen's urea agar according to (Cruickshank *et al*., 1975).

3. Serotyping of *Salmonella* species

All *Salmonella* isolates were subjected to serological typing by slide agglutination test in serological unit, Animal Health Research Institute, Dokki, Giza according to Grimont and Weill (2007). Only fresh bacterial cultures from 24 hours age colonies onto nutrient agar media were used.

4. PCR techniques:

DNA extraction DNA extraction from pure isolates (five isolates of *S*. enteritidis and one isolate of *S*. typhimurium) and direct egg content was done using the QIAamp DNA mini kit (Qiagen S.A., Courtaboeuf Cedex, France) with modifications from the manufacturer's recommendations. Briefly, 200 µl of the sample suspension was incubated with

200 µl of AL Buffer for 10 min at 56˚C. After incubation, 200 µl of 100% ethanol was added to the lysate. The sample was then washed and centrifuged following the manufacturer's recommendations. Nucleic acid was eluted with 100 µl of elution buffer provided in the kit.

Oligonucleotide Primer: Primers used were supplied from Metabion, Germany are listed in a Table 1. Primers were utilized in a 25- µl reaction containing 12.5 µl of EmeraldAmp Max PCR Master Mix (Takara, Japan), 1 µl of each primer of 20 pmol concentration, 4.5 µl of water, and 6 µl of DNA template. The reaction was performed in a T3 Biometra thermal cycler. The products of PCR were separated by electrophoresis on 1.5% agarose gel (Applichem, Germany, GmbH) in 1x TBE buffer at room temperature using gradients of 5V/cm. For gel analysis, 20 µl of the products were loaded in each gel slot. Gelpilot 100 bp ladder (Qiagen) and Gene ruler 100 bp DNA ladder (Fermentas) were used to determine the fragments sizes. The gel was photographed by a gel documentation system (Alpha Innotech, Biometra) and the data was analyzed through computer software.

Table 1: Oligonucleotide primers used in the study and their cycling conditions.

Target gene	Primers sequences (5- to 3-)	Amplified segment (bp)	Primary Denaturation	Amplification (35 cycles)			Final extension	Reference
				Secondary denaturation	Annealing	Extension		
stn	TTG TGT CGC TAT CAC TGG CAA CC ATT CGT AAC CCG CTC TCG TCC	617	94˚C 5 min.	94˚C 30 sec.	59˚C 40 sec.	72˚C 45 sec.	72˚C 10 min.	**Murugkar *et al*., 2003**
***inv*A**	GTGAAATTA TCGCCACGT TCGGGCAA TCATCGCAC CGTCAAAG GAACC	284	94˚C 5 min.	94˚C 30 sec.	55˚C 40 sec.	72˚C 45 sec.	72˚C 7 min.	**Olivera *et al*., 2003**
***avr*A**	CCT GTA TTG TTG AGC GTC TGG	422	94˚C 5 min.	94˚C 30 sec.	58˚C 40 sec.	72˚C 45 sec.	72˚C 10 min.	**Huehn *et al*., 2010**

RESULTS

Table 2: Incidence of *Salmonella* species in examined farm and balady egg content.

Items	No. of examined samples	Positive samples			
		Organism isolation*		Direct gene detection* *	
		No	%	No	%
Farm egg	200	2	1	0	0
Balady egg	200	6	3	2	1
Total	400	8	2	2	0.5

*Isolation of *Salmonella* species by conventional method then confirmed by PCR.
**Direct detection of virulent genes of *Salmonella* species from egg content samples by direct molecular identification.

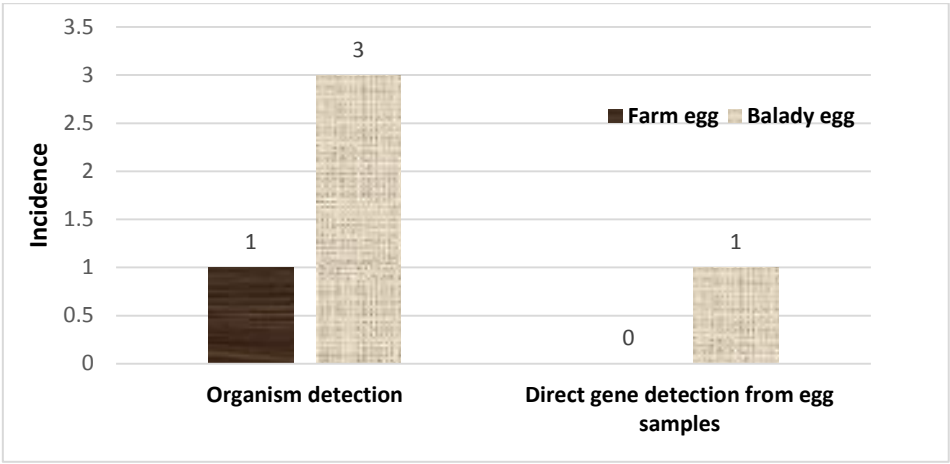

Figure 1: Detection of *Salmonella* species by conventional method and direct gene detection in egg content samples.

Table 3: Serotyping of *Salmonella* serovars isolated from examined farm and balady egg contents.

Serotyping	Farm eggs		Balady eggs		Antigenic structure
	No	%	No	%	
***Salmonella* enteritidis**	1	0.5	5	2.5	1,9,12: g,m
***Salmonella* typhimurium**	1	0.5	1	0.5	1,4,5,12:i:1,2

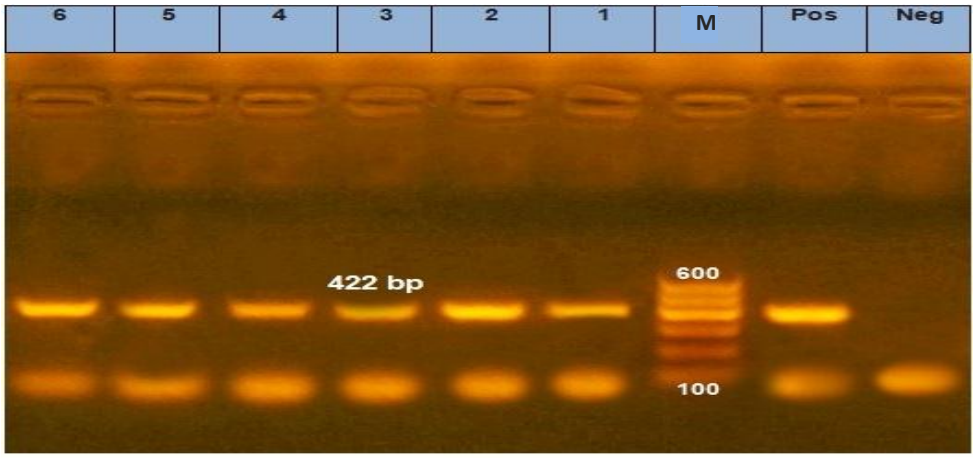

Figure 2: Agarose gel electrophoresis showing amplified PCR product of *avr*A gene. Lane M: 100 bp DNA Ladder, Lane 1-6: *Salmonella* isolates with *avr*A positive amplicons (422 bp)

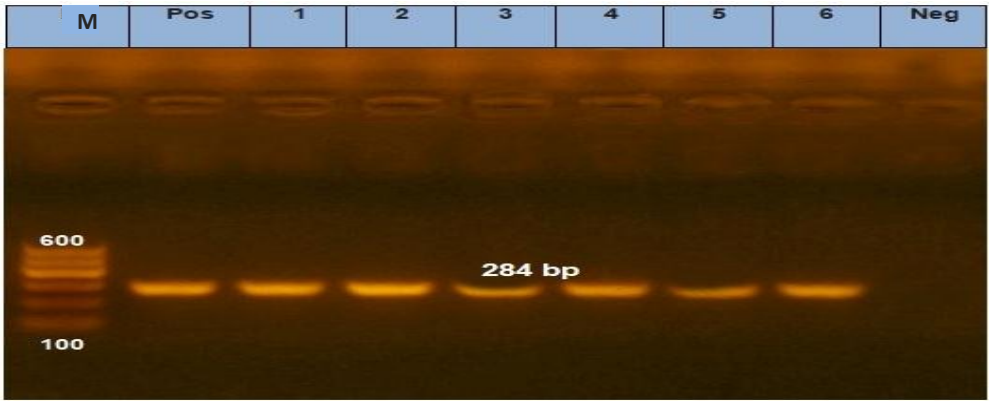

Figure 3: Agarose gel electrophoresis showing amplified PCR product of *inv*A gene. Lane M: 100 bp DNA Ladder, Lane 1-6: *Salmonella* isolates with *inv*A positive amplicons (284 bp)

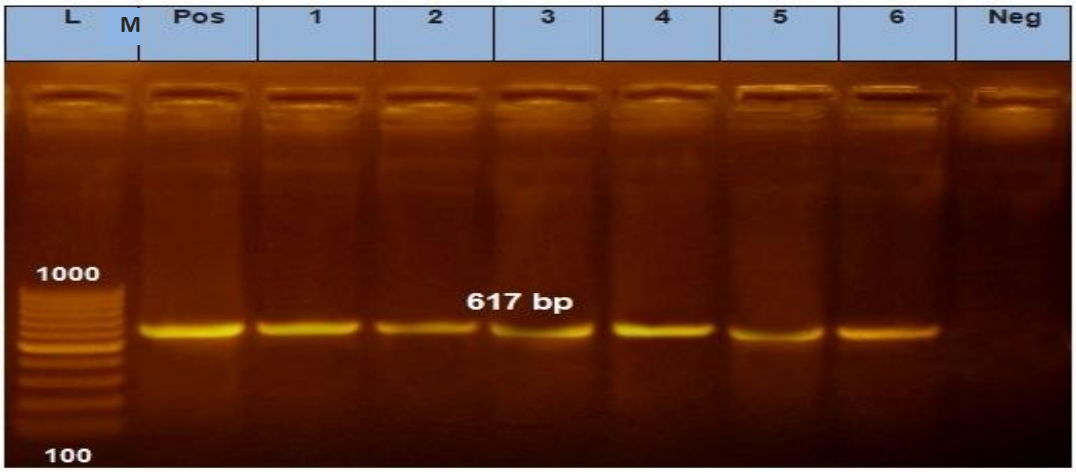

Figure 4: Agarose gel electrophoresis showing amplified PCR product of *stn* gene. Lane M: 100 bp DNA Ladder, Lane 1-6: *Salmonella* isolates with *stn* positive amplicons (617 bp)

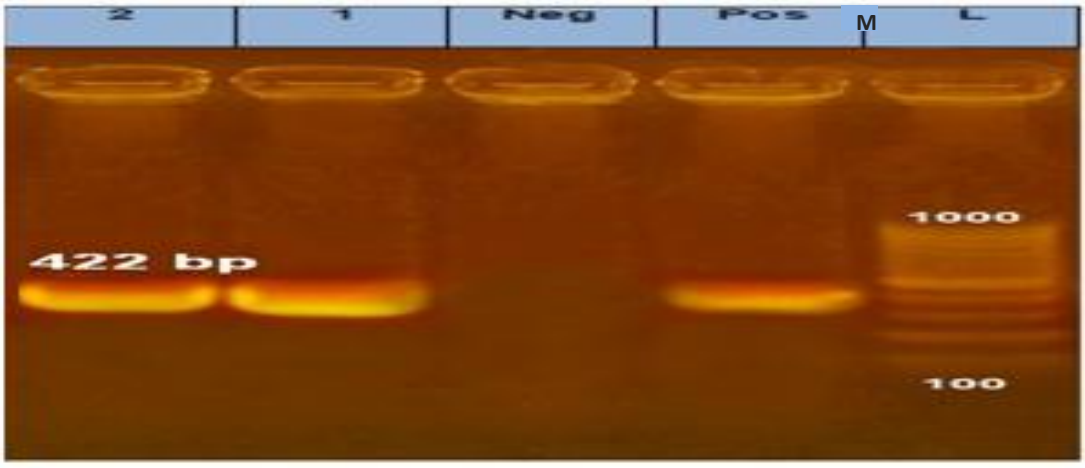

Figure 5: Agarose gel electrophoresis showing amplified PCR product of *avr*A gene from ten direct egg content samples. Lane M: 100 bp DNA Ladder, Lane 1-2: *Salmonella* isolates with *avrA* positive amplicons (422 bp)

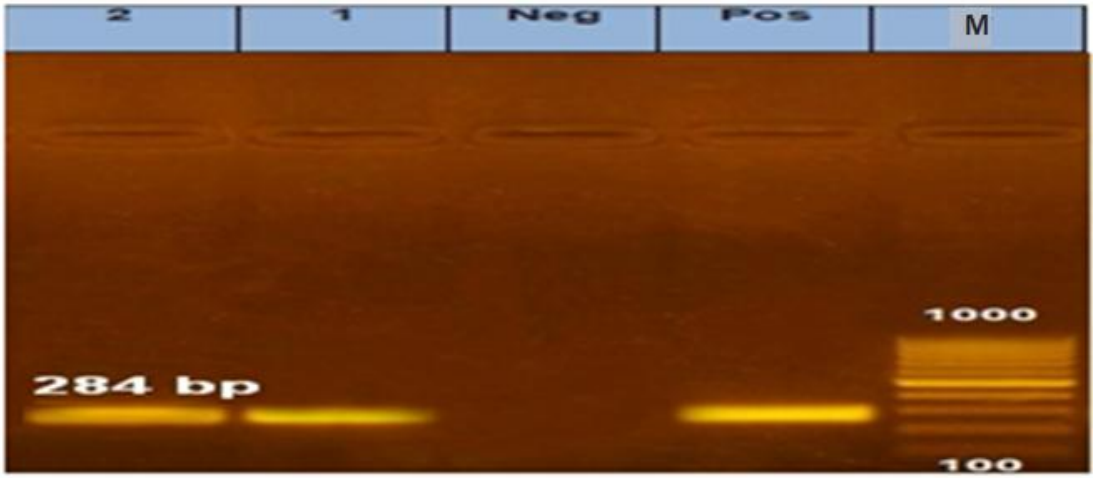

Figure 6: Agarose gel electrophoresis showing amplified PCR product of *inv*A gene from ten direct egg content samples. Lane M: 100 bp DNA Ladder, Lane 1-2: *Salmonella* isolates with *invA* positive amplicons (284 bp)

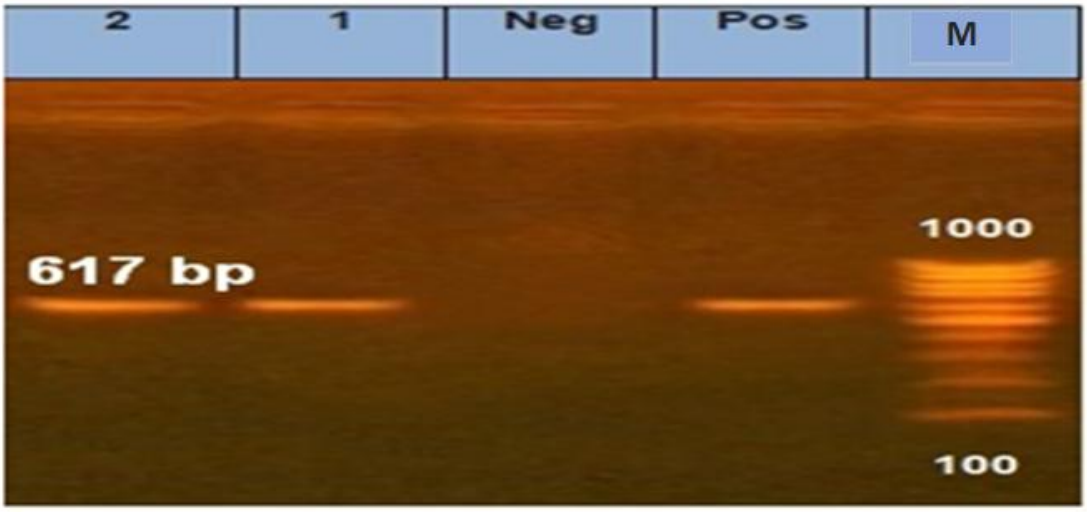

Figure 7: Agarose gel electrophoresis showing amplified PCR product of *stn* gene from ten direct egg content samples. Lane M: 100 bp DNA Ladder, Lane 1-2: *Salmonella* isolates with *stn* positive amplicons (617 bp)

DISCUSSION

Salmonella has the ability to persist on the surface of the egg shell and contaminate the content of egg when eggs are broken for preparation of food, which could pose a potential health risk to the society. Therefore, removal of the wastes and disinfection can greatly reduce *Salmonella* contamination on the shell and the content (Omwandho and Kubota, 2010).

The obtained results in Table 2 and figure 1 declared that the incidence of *Salmonella* in egg content of totally examined eggs was 2% (8/400) distributed as 2/200 (1%) in farm eggs, while incidence in balady eggs was 3% (6/200), this may be due to that balady eggs were more contaminated with faecal matter than farm eggs which have high level of hygienic measures during production. The aforementioned results revealed that incidence of *Salmonella* by direct gene detection using molecular identification was two (1%) from balady eggs while failed to be detected in farm eggs.

Nearly similar incidence of *Salmonella* in egg content was reported by Awny *et al*. (2018) who reported that incidence of *Salmonella* in egg contents of examined farm eggs was 4%. On the contrary, the same authors failed to detect *Salmonella* in egg contents of examined balady eggs. In addition, Zubair *et al.* (2017) failed to detect *Salmonella* in egg content of 350 examined eggs in Iraq. Also, Moosavy *et al.* (2015) could not isolate *Salmonella* from egg content of 150 examined commercial eggs collected from local stores in Iran.

EOSQC (2007) stated that the fresh table eggs should be free from *Salmonellae* species in their contents. According to these standards, the percentages of samples that failed to comply with the standards were 1 and 3% in farm and balady egg contents, respectively.

Contamination of egg shells represents a serious risk for the consumers, as they can directly infect and cross-contaminate the egg contents or other foodstuffs (Martelli and Davies, 2012). *Salmonella* may be able to contaminate egg contents by migration through the egg shell and membranes; such route, facilitated by moist egg shells, storage at ambient temperature and shell damage (Zeidler, 2002). The motility of *Salmonella* on egg shells may easily penetrate the shells to interior; the rate of penetration is influenced by humidity and storage temperature at which the eggs were produced and stored (Cox *et al*., 2000).

The obtained results in Table 3 revealed that serotyping of *Salmonella* species in examined farm eggs was one strain *Salmonella* enteritidis serotyped (1,9,12:g,m) and one strain *Salmonella* typhimurium serotyped *(*1,4,5,12:i:1,2). Meanwhile, in examined balady eggs were five strains (2.5%) *Salmonella* enteritidis serotyped as (1,9,12:g,m) and one strain (0.5%) *Salmonella* typhimurium serotyped as (1, 4, 5, 12:i:1, 2).

Historically, *S.* typhimurium is the most frequently serovars and S. enteritidis is the second as causative agents of human gastroenteritis throughout the world and were isolated from cases of food poisoning and represents about 50-60 of the cases (Sharma *et al*. 1996).

In the present study, both serovars of *S.* enteritidis and typhimurium were identified. Jamshidi *et al.*

(2010) could isolate *S*. enteritidis and Miranzadeh *et al*. (2012) could isolate *S*. typhimurium from eggs.

Salmonella enteritidis is the most prevalent serovar in the world (Betancor *et al*., 2010; Sasaki *et al*., 2011). Few eggs related outbreaks of salmonellosis caused by *S*. typhimurium were reported in humans in the European Union (3.5% against 77.2% caused by *S*. enteritidis (EFSA, 2010).

Figure 2 showed that, all 6 (100%) *Salmonella* isolates were found to carry *avr*A gene. The amplified PCR products of all *Salmonella* isolates on agarose gel electrophoresis yielded a 422 bp product in the *avr*A gene segment.

The virulence-associated effector protein *avr*A of *S.* enterica, which interferes with the first line of immune response of the target organism (Collier-Hyams *et al*., 2002), is an important partner in the virulence phenotype of this pathogen (Ben-Barak *et al*., 2006)

Presence of *inv*A gene confirms the invasive strains of *Salmonella* at the genus level. In the present study, all 6 (100%) *Salmonella* isolates were found to carry *inv*A gene. The amplified PCR products of all *salmonella* isolates on agarose gel electrophoresis yielded a 284 bp product in the *inv*A gene segment (Figure 3). It is speculated that strains without *inv*A gene are not invasive, or that they might be using other invasive mechanisms. However, their absence in *Salmonella* seems to be rare (Malorny *et al*., 2003).

The obtained data nearly agree with results obtained by Nagappa *et al*. (2007) who could isolated *Salmonella* typhimurium from eggs at incidence of 3% (3/100) in India, molecular identification using PCR revealed 3 isolates have *inv*A gene amplified at 284 bp.

Figure 4 revealed that, all 6 (100%) *Salmonella* isolates were found to carry *stn* gene. The amplified PCR products of all *Salmonella* isolates on agarose gel electrophoresis yielded a 617 bp product in the *stn* gene segment.

Recorded results were similar to that obtained by Naik *et al*. (2015) who revealed that *stn* gene was present in all the isolates (100%) irrespective of source of samples and region of sampling, *Stn* gene in *Salmonella* is highly conserved and it is expected to be a new target gene for detection of *Salmonellae* in field samples. *Salmonella* induced diarrhea is a complex phenomenon involving several pathogenic mechanisms including production of enterotoxin (Baloda *et al*., 1983) which is mediated by the *stn* gene (Chopra *et al*., 1987).

Most *S.* enterica strains (approximately 80%) contain the *avr*A gene as mentioned by Streckel *et al.* (2004). In addition, Diarra *et al*. (2014) found that the invasion gene (*inv*A) present in 97.9% of *Salmonella* enterica serovars and the heat-labile *Salmonella* enterotoxin (*stn*) serve as effector proteins, which are involved in the pathogenesis of salmonellosis, so the primer sets designed in the present study for *inv*A, *avr*A and *stn* genes allow simultaneous identification of all pathogenic *Salmonella* within the genus level.

Figures 5, 6 and 7 showing that two samples of five egg content samples from balady eggs were positive for target virulence gene including *avr*A, *inv*A and *stn* genes at molecular weight 422, 284 and 617 bp, respectively while failed to be detected from five farm egg content samples.

The use of *Salmonella* specific PCR with primers *inv*A is rapid, sensitive and more specific for detection of *Salmonella* in many food samples. The amplification of *inv*A gene has been validated as a standard for detection of invasion gene from *Salmonella* species (Ferretti *et al.*, 2001).

The PCR method using target gene remains a suitable molecular tool to diagnose Salmonella in animals. These findings have important health implications to the entire populace considering the high prevalence of virulence genes in food samples studied and it also underscores the need for rapid identification of Salmonella virulence genes using the PCR method (Stella *et al.*, 2015)

In general, egg-related outbreaks resulted from breakdowns in controlling measurements along the farm to fork continuum. International poultry control programs in developed countries have resulted in significant decreases in egg-related salmonellosis. These programs included; on-farm monitoring, diverting contaminated eggs for processing, culling infected flocks, cleaning and disinfection of sheds, maintaining cold chain of eggs, and vaccination of flocks (Moffatt and Musto, 2013). It is recommended that these controlling measurements should be done carefully in all countries including Egypt.

CONCLUSION AND RECOMMENDATION

From this study, *Salmonella* is an important causative agent for food poisoning outbreaks worldwide. The most common implicated food is egg or egg containing dishes. Eggs may be a source of transmission of Salmonellosis. *Salmonella* pathogenicity islands (SPIs) along with the virulence plasmids play an important role in survival and proliferation of bacteria in host system. PCR can be used as an alternative tool to the conventional

isolation and identification methods for the rapid detection of *Salmonellae*. Therefore, food handlers and the public should be encouraged to ensure good personal hygiene practices and proper cooking/food handling procedures through various trainings and education.

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التوصيف الجزيئي لأنواع السالمونيلا المعزولة من محتوي بيض دجاج المائدة

صابر علي سعد ، عبير حسن علي

E-mail: sabersaad284@yahoo.com Assiut University web-site: www.aun.edu.eg

السالمونيلا هي أحد أهم الميكروبات التي تنتقل عن طريق الأغذية التي يحصل عليها الإنسان وخصوصا البيض الخام أو الغير مطبوخ مما يسبب مشاكل صحية. لذا كان الهدف من هذه الدراسة هو عزل وتحديد أنواع السالمونيلا من محتويات البيض، بالإضافة إلى الكشف عن بعض جينات الضراوة مثل (*avrA*، *invA* و*Stn*) باستخدام تفاعل انزيم البلمرة المتسلسل من معزولات السالمونيلا وكذلك من عينات البيض مباشرة. ولذا تم جمع اربعمائة بيضة صالحة للأكل (٢٠٠ من بيض المزارع ٢٠٠ من البيض البلدي) من الأسواق المختلفة والمزارع في محافظة البحيرة. أظهرت النتائج التي تم الحصول عليها أن نسبة تواجد السالمونيلا كان ١ و٣٪ في بيض المزارع والبيض البلدي، على التوالي و١٪ بالكشف المباشر علي الجين المستهدف في البيض البلدي بينما لم يتم العزل من بيض المزارع. وكشف التصنيف السيرولوجى لمعزولات السالمونيلا أنه تم تحديد نوعين مختلفين من السالمونيلا وهما سالمونيلا انتريتيديس (عترة واحدة) وسالمونيلا تيفيميريم (عترة واحدة) من بيض المزارع بينما سالمونيلا انتريتيديس (٥ عترات) وسالمونيلا تيفيميوريم (عترة واحدة) من البيض البلدي. كما أنه تم الكشف عن بعض جينات الضراوة فى ست معزولات من السالمونيلا باستخدام تفاعل البلمرة المتسلسل، ووجد أن جميع معزولات السالمونيلا الستة تحتوي على جين *avrA* عند وزن جزئ ٤٢٢، وجين *invA* عند وزن جزئ ٢٨٤ وجين Stnعند وزن جزئ ٦١٧. وبالكشف المباشرعن بعض جينات الضراوة فى عشرة عينات من محتوي بيض المائدة(خمسة من بيض المزارع وخمسة من البيض البلدي) باستخدام تفاعل انزيم البلمرة المتسلسل، ووجد اثنتان فقط من بيض المائدة البلدي تحتوي على جين *avrA* عند وزن جزئ ٤٢٢، وجين *invA* عند وزن جزئ ٢٨٤ وجين Stnعند وزن جزئ ٦١٧.