# Detection of five major pathogenicity islands in *Salmonella* serovars isolated from broiler chicken

Asmaa El Sayed Mohammed El Sayed [1], Mohamed Wael Abdel-Azeem[2], Serageldeen Sultan[2], and Amany Abbas Yousif [1]

[1]Animal Health Research Institute, Sohag Branch, Sohag 82525, Egypt.
[2]Department of Microbiology, Faculty of Veterinary Medicine, South Valley University, Qena83523, Egypt.
dr_asmaa_lab@yahoo.com

**Abstract: Background:** *Salmonella enteritidis* and *Salmonella typhimurium* are the most prevalent serotypes in broiler chicken. These isolates possess a zoonotic impact to public health. PCR technique is sensitive, specific and useful in detection of pathogenicity islands genes to determine the virulence of *Salmonella* serovars. **Materials and Methods:** This study was conducted to determine the prevalence of five major *Salmonella* pathogenicity islands (SPIs) in *Salmonella* serovars isolated from broiler chicken in Sohag Governorate, Egypt. A total of 1000 samples were collected from 200 broiler chicken. The samples were intestine, liver, spleen, heart and yolk sac. **Results**: The colonial morphology, biochemical and serological identifications of the isolates revealed the presence of *Salmonella* in 32 out of 200 chicken (16%). The rate of recovery of *Salmonellae* from the different internal organs showed that high recovery rate was from intestine, liver, spleen, heart then yolk sac as the follow (35%), (23%), (16%), (14%) and (12%) respectively. *Salmonella enteritidis* and *Salmonella typhimurium* were the most prevalent serotypes (34.7%, 18.4% respectively) followed different *Salmonella* serotypes; *Salmonella kentucky*, *Salmonella virchow*, *Salmonella newport*, *Salmonella infantis*, *Salmonella anatum* and *Salmonella chester* (12.2%, 8.1%,8.1%,8.1%, 6.2% and 4.2%) respectively. PCR assay was carried out to detect *InvE*/A for SPI-1, *ssa*Q for SPI-2, *mgt*C for SPI-3, *spi4*R for SPI-4 and *sop*B for SPI-5 in all *Salmonella* isolates. *Inv*E/A, *ssa*Q, *mgt*C and *sop*B genes of the four pathogenicity islands, are detected in all *Salmonella* serovars isolated from broiler chicken. While *spi4*R was present in all *Salmonella* serotypes except *Salmonella infantis* this may due to deletion or mutation. **Conclusion:** PCR technique is sensitive, specific and useful in detection of pathogenicity islands genes to determine the virulence of *Salmonella* serovars. *Salmonella* (*enteritidis, typhimurium, kentucky, anatum, newport, virchow* and *chester*) carried all the virulence genes of the five pathogenicity islands. So these serovars can be considered as a fully virulent serovars.

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**Keywords:** *Salmonella*, Broiler, pathogenicity islands, Virulence Genes

## 1. Introduction

*Salmonella* infection is one of the most serious problems that affect poultry industry causing high economical losses not only due to high mortality in young chickens but also for the debilitating effect which predisposes for many other diseases. Poultry products have always topped the incidence of salmonellosis in many developing countries including India, Egypt, Brazil and Zimbabwe (Yang *et al.*, 2011). Salmonellosis is an important health problem and a major challenge worldwide. *Salmonella spp.* are recognized as the most causative agents of food poisoning (Gallegos-Robles *et al.*, 2008).

Poultry are commonly infected with a wide variety of *Salmonella enterica* serovars. Europian Food Safety Authority (EFSA, 2007) accounted *Salmonella enteritidis* and *typhimurium* for most zoonotic salmonellosis associated with food of animal origin (Collard *et al.*, 2008). The detection of *Salmonella* spp. in food is one of the most effective means of preventing salmonellosis (Favrin *et al.*, 2003). conventional culture methods for *Salmonella* detection require three to four days to obtain a negative result and up to seven days to get confirmed positive result, which is tedious, time consuming, labor-intensive and are not adapted for monitoring food products (Trevanich *et al.*, 2010). Since 1990's, several chromogenic media have been developed to detect *Salmonella*, such as Rambach, Salmonella Identification agar (SM-ID), CHROM agar, ABC Medium, Chromogenic Salmonella esterase agar, and Rainbow Salmonella Agar. They are based on a combination of biochemical characteristics and are highly specific (Rall *et al.*, 2005). Polymerase chain reaction (PCR) provides a new strategy for the detection of *Salmonella*. Studies of gene expression are extremely important to the understanding of numerous bacterial processes including virulence (Maharjan *et al.*, 2006).

*Salmonella* pathogenicity islands are clusters of chromosomal virulence genes found only within the genus *Salmonella* and are absent in non-pathogenic strains (Parkhill *et al.*, 2001). They are portions of DNA that have been acquired from other microorganisms by horizontal transfer. To date, twelve SPIs have been identified in different serovars, but only five are well characterized (Hensel, 2004).

Both SPI1 and SPI2 encode type three secretion system (T3SS) that translocate effector molecules into host cells. SPI-1 is required for invasion of host cells and induction of macrophage apoptosis, SPI-2 is required for systemic infection and replication within macrophages (Foley and Lynne, 2008). SPI-3 is required for survival in macrophages and the ability of *Salmonella* to grow in low-magnesium environments (Leung *et al.*, 2011), SPI-4 is required for intramacrophage survival and harbors genes for toxin secretion and apoptosis (Bingle *et al.*, 2008), SPI-5 has been found to cluster genes that encode multiple T3SS effector proteins that are secreted by the SPI-1 and/or SPI-2 T3SS (Amavisit *et al.*, 2003).

## 2. Material and Methods

### 2.1. Sample Collection and preparation:

A total of 200 broiler chickens were collected for *Salmonella* isolation. Using sterile scissors, intestine, liver, heart, spleen and yolk sac were individually cut into smallpieces, aseptically 25 g from each of intestine, liver and heart and 1 g from each of yolk sac and spleen was weighed into sterile stomacher bag (Al-Nakhli *et al.*, 1999).

### 2.2. Isolation of *Salmonella*:

According to (ISO, 2002) method each sample was inoculated separately in buffer peptone water and incubated at 37°C for not more than 18 hrs. 0.1 ml of culture obtained from pre- enrichment step was transferred to tube contain 10 ml of Rappaport Vassiliadis broth and incubate at 42°C for 24 hrs. Then a loopful from selective enriched media was streaked onto Salmonella chromogenic agar and incubated at 37°C for 24 hrs. Typical colonies on Salmonella Chromogenic Agar appeared as mauve colonies while *Enterobacteriaceae* other than *Salmonella* including E-*coli* appeared as blue, while most of *Proteus* spp. appeared colorless with or without brownish precipitation.

### 2.3. Identification of *Salmonella* Isolates

#### 2.3.1. Microscopic Examination:

Films from suspected purified colonies were prepared, fixed and stained with Gram stain.

#### 2.3.2. Biochemical Identification:

According to (ISO, 2002) method: Purified isolates were examined by oxidase, urease and TSI tests. Isolates gave negative both oxidase and urease and gave K/A with H2S and gas on TSI were confirmed with Analytical profile index 20 E (API20E). An API 20E strip was inoculated for each isolate and incubated at 37°C for 24 hr. Positive results were evaluated for each of the 20 biochemical tests. The seven-digit number was calculated, and the organism identity was determined using the profile manual or by calling the API Voice.

#### 2.3.3. Serological Identification:

*Salmonella* isolates were serotyped by the slide agglutination method using O and H antisera (Difco, Detroit, USA), according to the manufacturer's instructions.

### 2.4. Detection of SPIs Genes by PCR

PCR assay was carried out to detect *InvE*/A for SPI-1, *ssa*Q for SPI-2, *mgt*C for SPI-3, *spi4*R for SPI-4 and *sop*B for SPI-5 in all *Salmonella* isolates. PCR primers used for amplification of virulence genes are listed in (table 1).

**Table (1): The oligonucleotide primers used for PCR**

| Target Gene | Location | Oligonucleotide sequence (5′→ 3′) | Product size (bp) | References |
|---|---|---|---|---|
| *Inv*E/A (F) | SPI-1 | **5′** TGCCTACAAGCATGAAATGG **3′** | 450 | (Sánchez-Jiménez *et al.*, 2010) |
| *Inv*E/A (R) | | **5′**AAACTGGACCACGGTGACAA **3′** | | |
| *ssa*Q (F) | SPI-2 | **5′** GAATAGCGAATGAAGAGCGTCC **3′** | 677 | (Soto *et al.*, 2006) |
| *ssa*Q (R) | | **5′** CATCGTGTTATCCTCTGTCAGC **3′** | | |
| *mgt*C(F) | SPI-3 | **5′** TGACTATCAATGCTCCAGTGAAT**′3′** | 655 | (Sánchez-Jiménez *et al.*, 2010) |
| *mgt*C (R) | | **5′**ATTTACTGGCCGCTATGCTGTTG **3′** | | |
| *spi4*R (F) | SPI-4 | **5′** GATATTTATCAGTCTATAACAGC **3′** | 1269 | (Sánchez-Jiménez *et al.*, 2010) |
| *spi4*R (R) | | **5′** ATTCTCATCCAGATTTGATGTTG **3′** | | |
| *Sop*B (F) | SPI-5 | **5′** GATGTGATTAATGAAGAAATGCC **3′** | 1170 | (Soto *et al.*, 2006) |
| *Sop*B (R) | | **5′** GCAAACCATAAAAACTACACTCA **3′** | | |

DNA was extracted using QIAamp DNA Mini Kit according to the instructions of the manufacturer. Detection of virulence genes was performed by PCR conditions listed in (table 2). PCR performed in A 200 Gradient Thermal cycler (Japan). PCR products were separated by gel electrophoresis in 1.5% agarose in

Tris–acetate–EDTA (TAE) buffer at 100 V. And Solis BioDyne 100 bp ladder was included in each agarose run, and visualized by UV light illumination Gel documentation system (UVP Photo Doc) U.K.

**Table (2): Cycling conditions of the different gene amplification by PCR**

| Cycling condition | *Inv*E/A (SPI-1) | *ssa*Q (SPI-2) | *mgt*C (SPI-3) | *Spi4*R (SPI-4) | *Sop*B (SPI-5) |
|---|---|---|---|---|---|
| Initial denaturation | 95□C, 2 min. | 95□C, 2 min. | 95□C, 2 min. | 95□C, 2 min. | 95□C, 2 min. |
| Denaturation | 95□C, 1 min. | 95□C, 1 min. | 95□C, 1 min. | 95□C, 1 min. | 95□C, 1 min. |
| Annealing | 51□C, 1 min. | 58□C, 1 min. | 54□C, 1 min. | 51□C, 1 min. | 53□C, 1 min. |
| Extension | 72□C, 1 min. | 72□C, 1 min. | 72□C, 1 min. | 72□C, 1 min. | 72□C, 1 min. |
| Final extension | 72□C, 5 min. | 72□C, 5 min. | 72□C, 5 min. | 72□C, 5 min. | 72□C, 5 min. |
| No. of cycles | 30 | 30 | 30 | 30 | 30 |
| Storage | 8□C | 8□C | 8□C | 8□C | 8□C |

## 3. Results

Out of 200 examined broiler chicken there were 32 chicken positive for *Salmonella* with percentage 16% (32/200) and *Salmonella* was recovered from 49 internal organs out of 1000 examined samples with the percentage of 4.9% (49/1000).

The rate of recovery of *Salmonella* from internal organs was depicted in (table 3), Out of the 49 positive samples, the highest recovery rate of *Salmonella* isolates was found in intestine (35%) followed by liver (23%); spleen (16%); heart (14%) and lastly yolk sac (12%).

**Table (3): Recovery rate of *Salmonella* from internal organs of broiler chickens.**

| Examined organs | No. of positive samples | Percentage |
|---|---|---|
| Intestine | 17 | 35% |
| Liver | 11 | 23% |
| Spleen | 8 | 16% |
| Heart | 7 | 14 % |
| Yolk sac | 6 | 12% |
| Total | 49 | 100% |

The methods of *Salmonella* isolation are listed in (table 4). All samples were submitted for bacteriological examination using Salmonella chromogenic agar and obtained isolates suspected to be *Salmonella* 72/1000 were further identified by conventional biochemical tests (TSI, urease and oxidase) 57 gave typical *Salmonella* profile with 15 false positive organisms. These false positive organisms were *Proteus mirabilis* (4), *Proteus vulgaris* (3), *Citrobacterfreundii* (3), *Pseudomonas aeruginosa* (2), *Escherichia coli* (1), *Enterobacter aerogenes* (1) and *Klebsiella pneumoniae* (1).

Out of 57 positive *Salmonella* by conventional biochemical tests, only 50 samples were positive by API 20E which gave typical *Salmonella* profile and yielded 7 false positive organisms. These false positive organisms were *Klebsiella Pneumoniae* (3), *Pseudomonas luteola* (2), *Escherichia coli* (1), *Serratialiquefaciens* (1).

Out of 50 positive *Salmonella* by API 20E, 49 samples were confirmed as *Salmonella* by slide agglutination test with only one sample was atypical *Salmonella*.

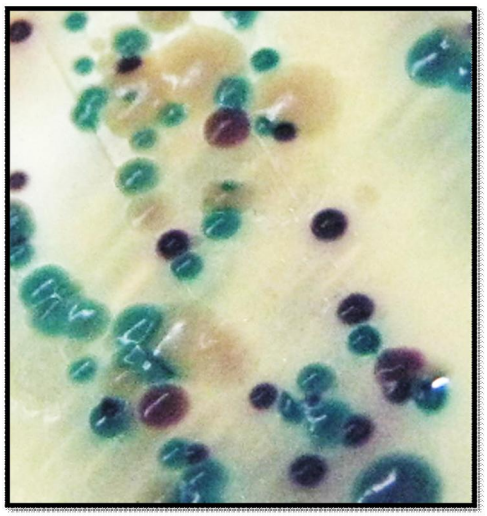

**Photo (1):** Colonies of *Salmonella* and other *Enterobacteriaceae* on Salmonella chromogenic agar. *Salmonella* appeared as mauve colonies while *Enterobacteriaceae* other than *Salmonella* appeared as blue, E-*coli* as blue-green, while *Proteus* as colorless with brownish precipitation.

**Table (4): The isolation of *Salmonella* spp. using sequence methods of identification: Salmonella chromogenic agar, Conventional biochemical reactions, API20E and Serological test**

| Method of identification | No. of tested samples | No. of positive samples ( Percentage) |
|---|---|---|
| Salmonella chromogenic agar | 1000 | 72 (7.2%) |
| Biochemical tests | 72 | 57 (79%) |
| API 20E | 57 | 50 (87.7%) |
| Serological tests | 50 | 49 (98%) |

The results of serotyping of isolated *Salmonella* species are listed in (table 5). 49 *Salmonella* isolates were serotyped using "O" and "H" antisera, serotypes recovered were 17 *Salmonella enteritidis* (34.7%), 9 *Salmonella typhimurium* (18.4%), 6 *Salmonella kentucky* (12.2%), 8.1% for each *Salmonella Virchow*, *Salmonella newport* and *Salmonella infantis*, 3 *Salmonella anatum* (6.2%) and 2 *Salmonella chester* (4.2%).

These *Salmonella* serotypes then examined for the presence of *InvE*/A for SPI-1, *ssa*Q for SPI-2, *mgt* C for SPI-3, *spi4*R for SPI-4 and *sop*B for SPI-5 by conventional PCR.

**Table (5): Serotyping of isolated *Salmonella* species**

| Identified strains | Group | Antigenic structure | | Frequency | Percentage |
|---|---|---|---|---|---|
| | | H | O | | |
| *Salmonella enteritidis* | D1 | g,m : 1,7 | 1,9,12 | 17 | 34.7% |
| *Salmonella typhimurium* | B | i : 1,2 | 1,4,5,12 | 9 | 18.4% |
| *Salmonella kentucky* | C3 | i : Z6 | 8,20 | 6 | 12.2% |
| *Salmonella virchow* | C2 | r : 1,2 | 6,7,14 | 4 | 8.1% |
| *Salmonella newport* | C2 | e,h:1,2 | 6,8 | 4 | 8.1% |
| *Salmonella infantis* | C1 | r:1,5 | 6,7 | 4 | 8.1% |
| *Salmonella anatum* | E1 | e,h:1,6 | 3,10 | 3 | 6.2% |
| *Salmonella chester* | B | e,h : e,n,x | 1,4,5,12 | 2 | 4.2 % |

**Photo (2):** *Salmonella* identification on API 20E

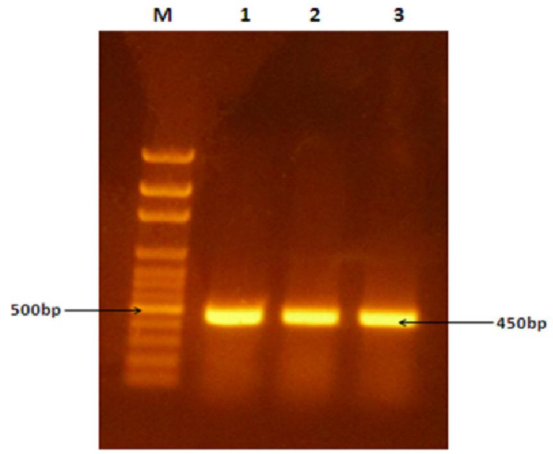


**Photo (3):** Agarose gel electrophoresis of ampilifie I*nv*E/A PCR product (450bp). Lane M: 100 bp DNA ladder. Lane 1, 2&3 examined *Salmonella* spp. positive for *Inv*E/A (SPI-1).

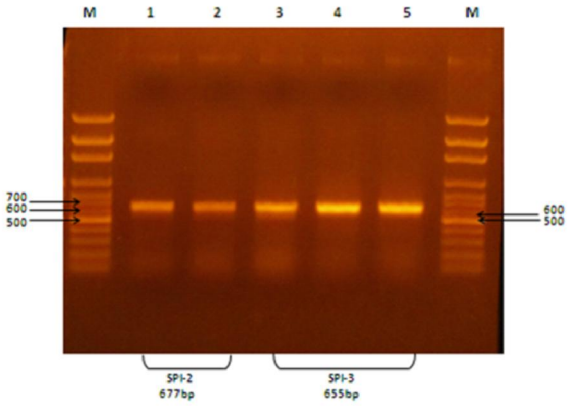


**Photo (4):** Agarose gel electrophoresis of ampilified *ssa*Q PCR product (677bp) and *mgt*C (655bp). Lane M: 100 bp DNA ladder. Lane 1& 2 examined *Salmonella* spp. positive for *ssa*Q (SPI-2). Lane 3,4&5 examined *Salmonella* spp. positive for *mgt*C (SPI-3).

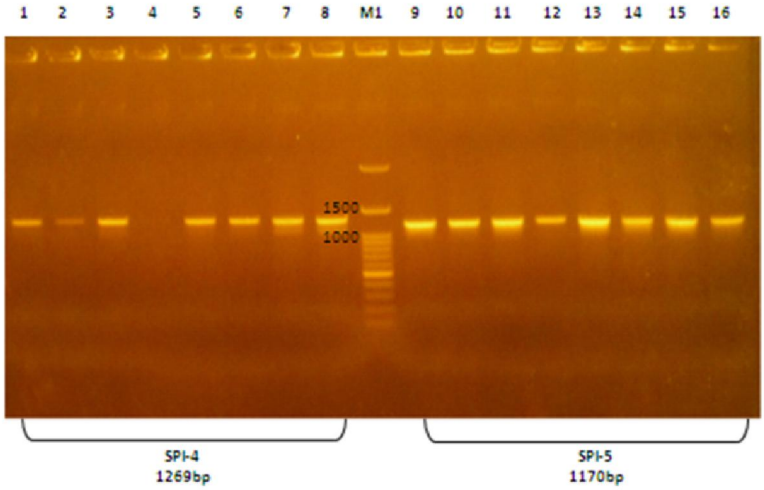


**Photo (5):** Agarose gel electrophoresis of ampilified *spi4*R PCR product (1269bp) and *Sop*B (1170bp). Lane M1: 100 bp plus DNA ladder. Lane 1, 2,3,5,6,7&8 examined *Salmonella* spp. positive for *spi4*R (SPI-4). Lane 4 *Salmonella Infantis* negative for *spi4*R (SPI-4). Lane 9,10,11,12,13,14,15&16 examined *Salmonella* spp. positive for *Sop*B (SPI-5).

The result indicate that *Inv*E/A, *ssa*Q, *mgt*C and *sop*B genes of the four pathogenicity islands, are detected in all *Salmonella* serovars. While *spi4*R was present in all *Salmonella* serotypes except *Salmonella infantis* this may due to deletion or mutation.

## 4. Discussion

*Salmonella* infection is one of the most important bacterial diseases in poultry causing heavy economic loss through mortality and reduced production. In the present study, the incidence of *Salmonella* in broilers was 16% (32out of 200 chicken). This is in agreement with the results obtained by (Mohamed *et al.*, 2009) who found that 14% of broilers were positive for *salmonella*. However, (Antunes *et al.*, 2003) reported that *Salmonella* present with higher incidence in (60%) of the examined chicken. The difference in the prevalence rates may be due to socioeconomic factors.

Recovery of *Salmonella* species from internal organs of the examined chickens were higher from intestine 35% followed by liver, spleen, heart and yolk sac 23%, 16%, 14% and 12% respectively. It was clear from these results that higher isolation rate of *Salmonella* species was from intestine. Intestine contained the highest numbers of *Salmonella* bacteria (Carrique-Mas and Davies, 2008). The intestine is the best overall organs to culture for *Salmonella* (Stecher *et al.*, 2007). While (Ezzat *et al.*, 2014) recovered *Salmonella* species with higher rate from liver followed by caecum, spleen, heart and kidney 9.5%, 5.5%, 4.5%, 3% and 2% respectively.

Serological identification of isolated *Salmonella* species revealed that *Salmonella enteritidis* and *Salmonella typhimurium* were the most prevalent serotypes (34.7%, 18.4% respectively) followed different *Salmonella* serotypes; *Salmonella kentucky*. *Salmonella* virchow, *Salmonella newport*, *Salmonella infantis*, *Salmonella anatum* and *Salmonella chester* (12.2%, 8.1%, 8.1%, 8.1%, 6.2% and 4.2% respectively). These are consistent with (Abo-shama, 2013) and (Ahmed and Shimamoto, 2014) they reported that *Salmonella enteritidis* and *typhimurium*

were the most prevalent serotypes in the examined samples. While (Roy *et al.*, 2002) isolated *Salmonella kentucky* and *Salmonella enteritidis* with percentage of 21.64% and 5.15%, respectively.

Salmonella chromogenic agar used in this study yielded high degree of sensitivity 100% this may have resulted from a better separation of colonies on Salmonella chromogenic medium, where there was an easy distinction of magenta from blue or colorless colonies (Cassar and Cuschieri, 2003). The same result obtained by (Maddocks *et al.*, 2002). While the specificity of Salmonella chromogenic medium was 98.4% (low number of false positives) nearly similar to and (O'Neill *et al.*, 2003) and higher than (Maddocks *et al.*, 2002) due to the difference in a composition of each chromogenic medium (van Dijk *et al.*, 2009).

Most false-positive organisms on Salmonella chromogenic agar in this study were due to *Proteus* and *Citrobacter*species. This was due to the large number of *Proteus* and *Citrobacter* in the samples (Perry and Freydiere, 2007). False positive colonies that were not *Proteus* or *Citrobacter* were mainly *Pseudomonas aeruginosa* or other small Gram negative rods same results obtained by (Gaillot *et al.*, 1999). These colonies of *Pseudomonas* species were easily differentiated from *Salmonella* by the oxidase test (Cassar and Cuschieri, 2003). All *E. coli* appeared as distinct blue colonies Interestingly, there was only one strain of *E. coli* produced mauve colonies on Salmonella chromogenic agar which required additional biochemical testing to definitively identify it (Maddocks *et al.*, 2002).

API 20E is a diagnostic test for *Salmonella*, based on identifying the biochemical properties of the bacteria, has been the standard test for *Salmonella* detection in many diagnostic laboratories (Nucera *et al.*, 2005). Previous studies of API 20E have reported both good (Peele *et al.*, 1997) and inaccurate (Robinson *et al.*, 1995). Certain groups of organisms including *Salmonella* were found to give low correlation on API 20E test when using primary isolation MacConkey plates in the clinical laboratory (Aldridge *et al.*, 1978). In this study we used API 20E as a confirmatory method after conventional biochemical tests.

Detection of virulence genes (virulotyping) by PCR technique recently has shown to be a useful tool for the characterization of *Salmonella* isolates (Huehn *et al.*, 2010). In this study we investigated the presence of genes of the pathogenicity islands (SPI-1 to SPI-5), in *Salmonella* isolates recovered from broiler chicken.

The chromosomally located invasion (*Inv*A) gene is thought to trigger the invasion of *Salmonella* into cultured epithelial cells (Darwin and Miller, 1999). In this study, PCR assay was carried out for the detection of the *Inv*A gene from isolated strains has revealed that the gene was present in all of the isolates (100%) that was demonstrated by the presence of a 450 bp PCR amplified fragment. The results obtained in the present study were in corroboration with (Amin and El Rahman, 2015).

*ssa*Q gene implicated in the secretion system apparatus protein of second T3SS encoded with (SPI-2), this genetic element has a central role in systemic infections by *Salmonella* spp. and intracellular pathogenesis (Bugarel *et al.*, 2011). *mgt*C magnesium transport protein is a putative P-type ATPases which encodes a membrane protein that is important for full virulence in the mouse (Günzel *et al.*, 2006). PCR assay was carried out for the detection of *ssa*Q gene and *mgt*C from isolated strains has revealed that both genes were present in all of the isolates (100%). The results obtained in the present study were in corroboration with (Huehn *et al.*, 2010; Osman *et al.*, 2014).

*SPI-4*R gene is antibiotic ABC transporter ATP-binding protein confined to SPI-4 which has been suspected to play a role in the invasion of cultured epithelial cells (Guiney and Fierer, 2011). PCR assay was carried out for the detection of *SPI-4*R gene from isolated strains has revealed that the gene was present in all of the isolates except *Salmonella infantis* was absent. (Bhowmick *et al.*, 2011) suggested that negative amplification result of virulence associatedgenes might be either due to simple point mutations or the absence of the gene due to insertions or deletions.

*Sop*B gene implicated in the translocated effector protein of T3SS for SPI-5 translocated into the host cytosol, where it mediates inflammation and fluid secretion in intestinal mucosa (Bugarel *et al.*, 2011). PCR assay was carried out for the detection of the *Sop*B gene from isolated strains has revealed that the gene was present in all of the isolates (100%) that was demonstrated by the presence of 1170 bp PCR amplified fragment. The results obtained in the present study were in corroboration with (Osman et al., 2014).

## 5. Conclusion

*Salmonella enteritidis* and *Salmonella typhimurium* were the most prevalent serotypes in broiler chicken samples. These isolates possess a zoonotic impact to public health. Salmonella chromogenic agar is highly sensitive, specific and useful in *Salmonella* detection from chicken. API 20E is accurate, quick and simple and offer a reasonable alternative to conventional systems for identifying *Salmonella*. PCR technique is sensitive, specific and useful in detection of pathogenicity islands genes to determine the virulence of *Salmonella* serovars. *Salmonella* (*enteritidis*, *typhimurium*, *kentucky*,

*anatum*, *newport*, *virchow* and *chester*) carried all the virulence genes of the five pathogenicity islands. So these serovars can be considered as a fully virulent serovars.

**Corresponding Author:**
Dr. Asmaa El Sayed Mohammed El Sayed
Address: Animal Health Research Institute, Sohag Branch, Sohag 82525, Egypt.
Mobile no.:00201100121328 / 00201221964484.
E-mail: dr_asmaa_lab@yahoo.com

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