Journal of Current Veterinary Research

ISSN:4026-2636

Journal homepage: http://www.jcvr.journals.ekb.eg

Molecular Detection of Some Virulence Genes of *Salmonella* Serotypes Isolated From Poultry in Egypt

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ABSTRACT

Salmonellosis is recognized as an important health problem and a major challenge in poultry industry worldwide. This study highlights the prevalence rate of salmonella serotypes in chickens as well as demonstration of some critical virulence genes by using specific primers sets in PCR reaction. Out of 100 freshly dead chickens, sixteen chicken samples were positive to *Salmonella* with 16% prevalence rate.The rate of recovery of Salmonellae from the different internal organs showed that the intestine is the most predication seat followed by, heart and liver with (8%), (6%) and (2%), respectively. Salmonella isolates (16) from chickens were serotyping using O and H antigen. The results revealed that S**.** Enteritidis was the most serotype found in 5 isolates (31.25%) followed by *S. Infantis* in 4 isolates (25%). Both *S. Newlands* and *S. Kentucky* found in 2 isolates 12.5%. Each of *S. Ferruch*, *S. Weybridge* and *S. Naestved* have one isolate (6.25%).The application of PCR for detection of *inv*A, *avrA* and *stn* virulence genes in nine salmonella serovars revealed that these genes (invA, *avrA* and *stn)*were detected in 100% of tested strains except *S. Naestved* does not contain *stn* gene. Hence, this study recommends that PCR may be used for rapid and sensitive detection of *Salmonella* and help in understanding the role of these genes in pathogenicity of salmonella infection in poultry.

Keywords**:** *Salmonella, PCR, invA, stn, avrA gene, Serotypes****.***

INTRODUCTION

Salmonella serovars aredescribed as important causes of foodborne infections in humans, and frequent etiological bacterial agents of foodborne disease outbreaks. In particular, two *Salmonella* serotypes, S. *enteritidis* and *S. typhimurium* identified as major causes of human illness in the 1980s and1990s, with public health concern and the economy in industrialized countries (Bailey and Maurer, 2001). *Salmonella* is enteric bacteria organism have the ability to induce different illness in animals and human. Yet, there are annually new serovars being discovered although more than 2,500 serovars of *Salmonella* were present (Brenner et al., 2000). Human salmonellosis cases resulted mainly from consumption of raw or uncooked foods in particular, foods of poultry origin which considered one of the most listed foods type incriminated in human salmonellosis outbreaks in many countries such as Egypt, India, Brazil, and Zimbabwe (Yang et al., 2011). Several syndromes are caused by salmonella in different animal species. For examples, avian salmonellosis is a problem of economic concern in poultry industry from production stage till marketing. Many of Salmonella species carried several virulence factors that enhanced a critical role in pathogenicity and determine the method of transmittion as well as the severity of infection to the target hosts. These genes are found to be located in chromosomal segment loci called Salmonella pathogenicity island, (SPI) which determined to the success of pathogen as an intracellular pathogen (Hensel, 2004). It though that these genes help in maintenance the integrity of membrane composition of

Salmonella serotypes (Nakano et al., 2012). Of these genes, *Salmonella* enterotoxin gene that encoded *stn* was not located on SPIs. The enterotoxin induces more loss of intestinal fluids causing diarrhea. This enterotoxin is correlated in its function, immunologically and genetically to the toxin of *Vibrio cholerae* and *Escherichia coli* (Jay et al. 2003). Moreover, *Salmonella* strains may also produce heat labile cytotoxin which may exert damage to the surface of intestinal mucosal and leading to inflammation and enteric disease (Jay *et al*., 2003; Hanes 2003). Furthermore, *inv*A gene of salmonella can be used as target for detection of Salmonella by PCR which considered as rapid and sensitive technique (Shanmugasamy et al., 2011). The *inv*A target gene is unique and prevalent in all clinical strains of *Salmonella* (Galán, 1996) although this gene absents from other genera such as *Escherichia* (Bäumler et al., 1998).

MATERIAL AND METHODS

A total of 300 samples were collected form (liver, heart and intestine) of 100 freshly dead native chickens. Age of chickens ranged from one day to 4 weeks old. The collected samples then transferred in ice box as soon possible to laboratory for bacteriological examination.

Bacteriological isolation and biochemical identification of salmonella

Typical isolation of salmonella species starts with perenrichment of samples in buffered peptone water for 37C for 16 hours, followed by selective liquid broth on tetrathionate broth, then incubated at 37ºC for 18hrs and finally culturing in Salmonella Shigella agar (S-S agar) for 24hrs at 37ºC. Colonies showing typical appearance of pale colored colonies with or without black centers were stained with Gram's in accordance of (Quinn et al. 2002) to detect its morphological characteristics. Further biochemical properties were carried out as described by (ISO 6579: 2002).

Serological identification of *Salmonellae*

Serological identification of salmonella species was tested according to Kauffman – White scheme (Kauffman, 1974) to determine Somatic (O) and flagellar(H) antigens using Salmonella antiserum (DENKA SEIKEN Co., Japan).

Molecular detection of invA, avrA and stn genes of salmonella serotypes:

Table (1): Primers sequencing and cycling conditions of the different primers during cPCR

Gene	Sequence	Primary denaturation	Secondary denaturation	Annealing	Extension	No. of cycles	Final extension	Reference
invA	GTGAAATTATCGCCACGTTCGGG CAA TCATCGCACCGTCAAAGGAACC	94˚C 5 min.	94˚C 30 sec.	55˚C 30 sec	72˚C 30 sec	35	72˚C 5 min.	**Oliveira *et al*., 2003**
avrA	CCT GTA TTG TTG AGC GTC TGG AGA AGA GCT TCG TTG AAT GTC C	94˚C 5 min.	94˚C 30 sec.	58˚C 30 sec.	72˚C 30 sec.	35	72˚C 10 min.	**Huehn *et al*. 2010**
Stn	TTG TGT CGC TAT CAC TGG CAA CC ATT CGT AAC CCG CTC TCG TCC	94˚C 5 min.	94˚C 30 sec.	59˚C 45 sec.	72˚C 45 sec.	35	72˚C 10 min.	**Murugkr *et al*., 2003**

Table (2): Preparation of PCR Master Mix according to Emerald Amp GT PCR mastermix (Takara) Code No. RR310A kit in 25 µl

Component	Volume/reaction
Emerald Amp GT PCR mastermix (2x premix)	12.5 µl
PCR grade water	4.5 µl
Forward & Reverse primer (20 pmol)	1 µl for each
Template DNA	6 µl
Total	25 µl

Table (3) Incidence of the isolated *Salmonellae* from different internal organs of chickens.

Type of sam	Total number of examined or from 100 birds	Positive	
		NO.	%
Liver	100	2	2
Intestine	100	8	8
Heart	100	6	6
Total		16	16

*Percentage was estimated according to total number of birds (100)

RESULTS:

Results of serotyping of *Salmonellae* isolated fromchickens:

Salmonella isolates (16) from chickens which serotyping using O and H antigen. The results revealed that S**.** Enteritidis was the most serotype found in 5 isolates (31.25%) followed by *S. Infantis* in 4 isolates (25%). Both *S. Newlands* and *S. Kentucky* found in 2 isolates 12.5%. Each of *S. Ferruch, S. Weybridge* and *S. Naestved* have one isolate (6.25%).

Results of Polymerase chain reaction for detection of commonvirulence genes in *Salmonella* isolates:

Nine *salmonella* isolates were examined for detection of virulence genes (*invA*, *avrA* and *stn*) genes by conventional PCR. All examined isolates harbored the three genes except *S. Naestved* does not contain *stn* gene.

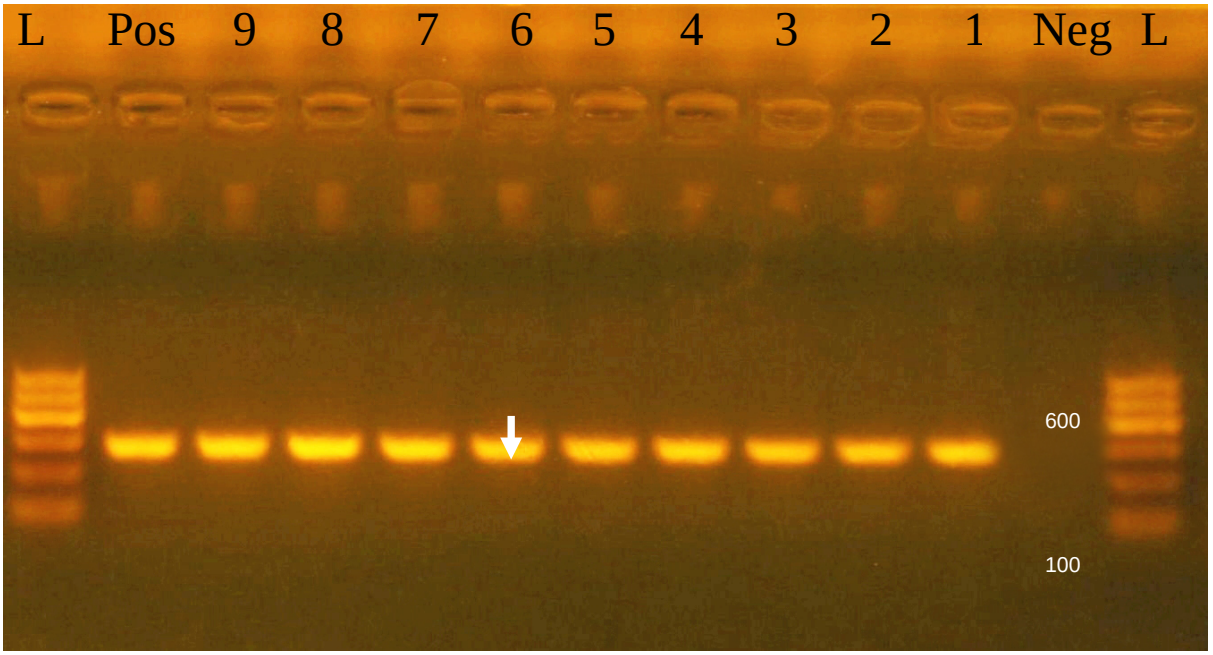

Photo (1): Agarose gel electrophoresis 1.5%showed the *invA* gene (284 bp): Lane L: 100-600pbDNA ladder.Pos.: Positive control. Neg.: Negative control. Lane 1-3: *S. Enteritidis*. Lane 4: S. Newlands. Lane 5: *S. Infantis*. Lane 6: S. Weybridge. Lane7: *S. Kentucky*. Lane8: *S. Naestved*. Lane9: *S. Ferruch*.

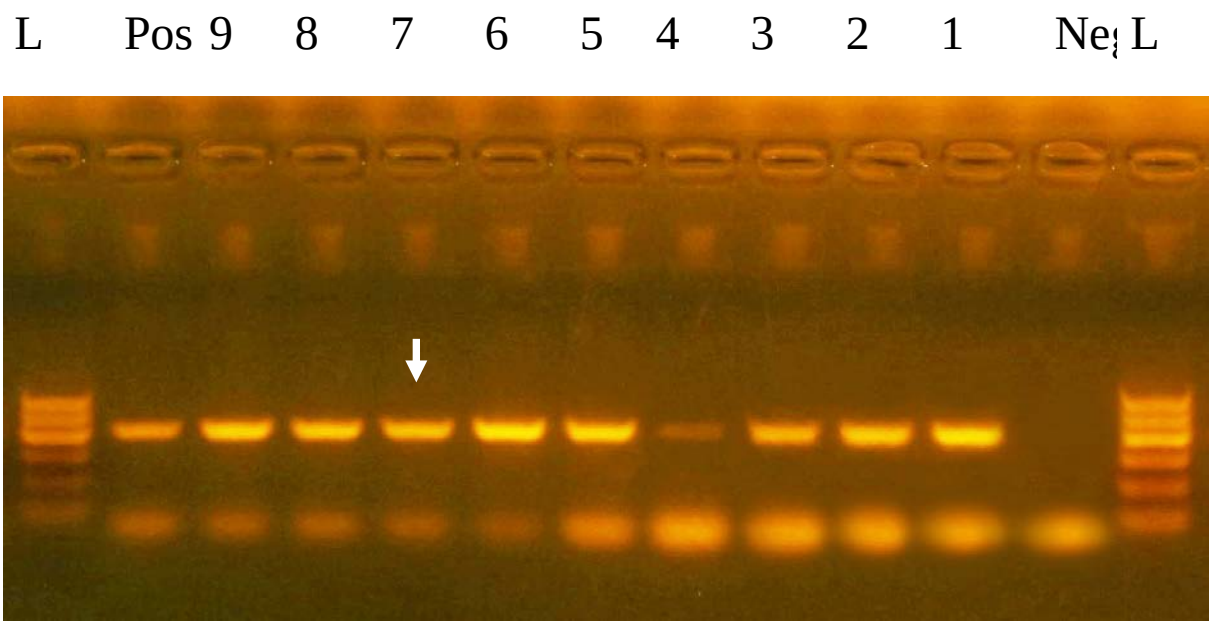

Photo (2): uniplex PCR for the detection of *avrA* gene (422 bp). Lane L: 100-600pb DNA ladder. Pos.: Positive control. Neg.: Negative control. Lane 1-3 *S. Enteritidis*; Lane 4: *S. Newlands*; Lane 5: *S. Infantis*; Lane 6: S. Weybridge; lane 7: *S. Kentucky*; Lane 8: S. Naestved.; lane 9: S. Ferruch

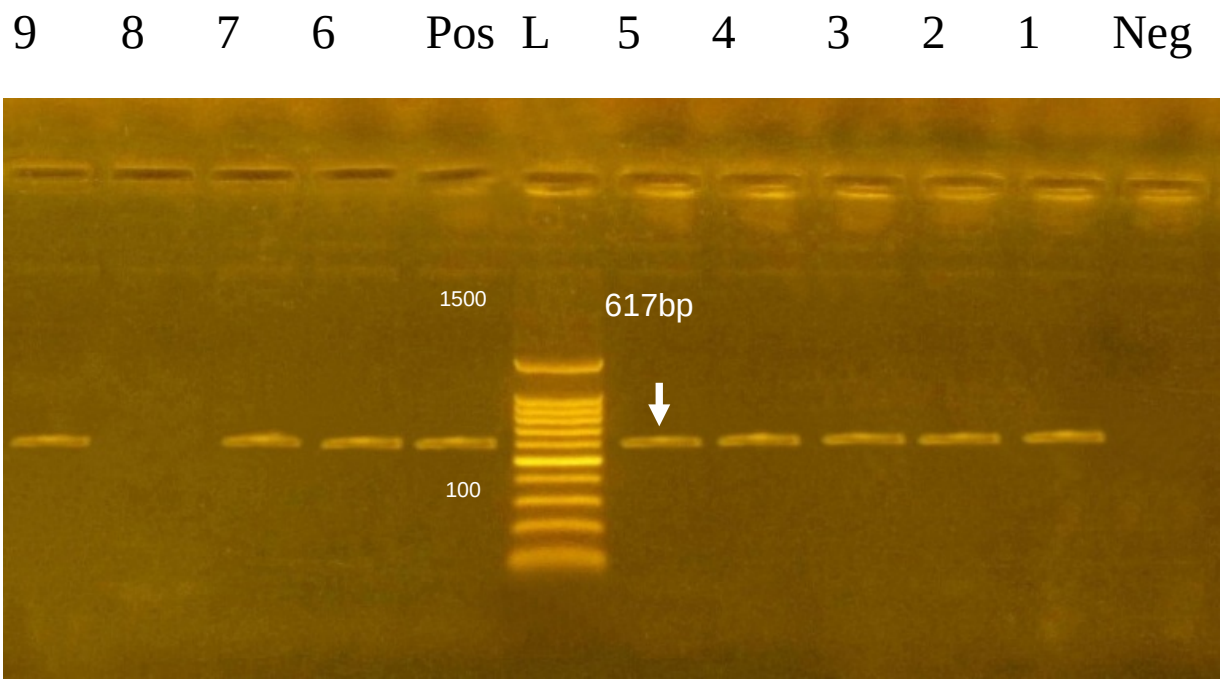

Photo (3): uniplex PCR for the detection of stn gene (617 bp): Lane L: 100-1500pb DNA ladder.Pos.: Positive control. Neg.: Negative control. Lane 1-3: *S. Enteritidis*.Lane 4: *S. Newlands*; Lane 5: *S. Infantis*; Lane 6: *S. Weybridge*; Lane7: *S. Kentucky*. Lane8: S. Naestved.; Lane9: S. Ferruch.

DISCUSSION:

Infection with Salmonella serotypes in poultry and humans has been dramatically increased worldwide resulting in severe economic loss and illnesses. Also it considered the major etiological agent involved in food-borne outbreaks (Majowicz et al., 2010; Scallan et al., 2011). The present study revealed that the over prevalence rate of salmonellosis in poultry was 16% while the prevalence rate from liver, heart and intestine were 2, 6, and 8% respectively. Our results were in close contact with (Ammar et al., 2016) who reported 17%rate of salmonella in Egypt. Higher prevalence rates (21.9% and 52.2%) were found in studies of (Rahman et al., 2004 andYang et al., 2011) in Bangladesh and China respectively. While lower percentage 2.7% was recorded in Brazil (Medeiros et al., 2011). This wide variation in prevalence may attributed to the difference in

methods of sampling, isolation and geographic distribution as well as source of samples. In this study serological identification of salmonella serovars was done as described by (Kauffman, 1974) and the most identified serotypes is S. Enteritidis 5 isolates (31.25%) followed by *S. Infantis* 4 isolates (25%). Both *S. Newlands* and *S. Kentucky* 2 isolates 12.5%. Each of *S. Ferruch*, *S. Weybridge* and *S. Naestved* have one isolate (6.25%). Previous studies in Egypt revealed that *Salmonella* Typhimurium (52.94%) was the most common identified serotypes (Ammar et al., 2016) . Moreover, S. Enteritidis and S. Typhimurium from chicken (58.33% and 41.66%, respectively (Nagwa et al., 2012). The*S.* Enteritidis, *S.* Typhimurium are the most common *Salmonella* serovars causing salmonellosis worldwide (2006, WHO). In Saudi Arabia, *S.* Enteritidis and *S.* Typhimurium dominated among the recovered *Salmonella* serovars from chicken (55.56% and 22.22%) respectively (Moussa et al., 2010).Concerning to the molecular detection of some virulence genes of salmonella serovars, this study examined nine *salmonella* isolates for detection of virulence genes (*invA*, *avrA* and *stn*) genes by conventional PCR. All examined isolates harbored the three genes (100%) except *S. Naestved* does not contain *stn* gene. The role of *invA* gene encodes as a protein structure in the bacterial membrane which was essential to invade the epithelial cells of intestine.(Darwin and Miller, 1999). Many studies detected *invA* gene in 100% of salmonella serovars as recorded in Egypt by (Karmi, 2013 and Ammar et al., 2016) and in international countries as in reports of(Samanta et al., 2014and Chaudhary et al., 2015) and with (91.0%) in Nigeria by (Anejo-Okopi et al., 2016).Many protein effectors of the type III secretion system (TTSSs) such as *avrA* and *sopE* usually involved in most of gram negative bacteria as salmonella species (Cornelis and Van Gijsegem, 2000)which responsible for impairment of IL-8 and TNF-α inflammatory cells and macrophages and subsequently increase bacterial virulence properties (Ben-Barak et al. 2006). Our results revealed that *avrA* gene was detected in all nine examined serotypes (100%). The same results was reported in studies (Hopkins and Threlfall 2004 and Borges et al., 2013) with 100% and 99% respectively.Lower frequencies was obtained in several studies (Rahman etal. 2004; Zou et al. 2011 and Liu et al. 2012). Moreover (Cesco,2010) detected *avrA* and *sopE* genes with 17.1% and 9.7% in *Salmonella* Hadar isolates, respectively. This variation in prevalence of these genes may be due to that the recombination of these genes locations (Hopkins and Threlfall 2004). These findings suggested that any changes in the proteins arrangement, such as *sopE* and *avrA*, may cause changes in the capability of this serovar to adapt to new hosts and, consequently, the emergence of novel virulent strains (Prager et al. 2000). Some authors hypnosis that the high frequency of *avrA* gene is more prevalent in pathogenic salmonella strains (Ben-Barak et al. 2006). Other researchers as (Suez et al. 2013) claimed that some of these proteins genes have an important role in *Salmonella* virulence. However, their absence in some isolates, such as *sopE*, suggests that they are not essential for infectivity of salmonella in the human host. Regarding to the results of *stn* gene, our results detected *stn* gene in all nine examined serotypes with 100%. These come in contact with (Rahman, 1999) who detected this gene in 100% of examined salmonella isolates. While (Ammar et al., 2016) used PCR for detection of *stn* gene in (58.82%).

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