Molecular Characterization of *Salmonella Enterica* Serovars Isolated From Chicken

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Abstract

The estimation of virulence and antibiotic resistance in Salmonella strains from chickens helps in mapping out suitable preventive ways to control the spread of this enteric microorganism in Egypt. Therefore this work was designed to study the molecular characterization of Salmonella enterica serovars isolated from different samples in chicken. Two hundred and seventy samples collected from different farms at Sharkia Governorate during the period from November 2015 to May 2016. All Salmonella isolates were analyzed for the presence of Salmonella serovars and for the presence of the most predominant virulence genes. The result revealed an incidence of Salmonella from faecal swabs and internal organs (38/270) with an overall incidence 14.07% from chicks only. Additionally, absolute resistance was observed for amoxicillin/clavulanic acid, ampicillin, doxycycline and ceftriaxone**.** On the other hand, sensitivity to ciprofloxacin (81.6%), gentamicin (78.9%) , sulphamethoxazol/trimethoprim (73.7%) ,amoxicillin (65.8%)and chloramphenicol(63.2%). All isolates of *Salmonella* were positive to *inv*A ,*hil*A , *avr*A, *bcf*C, *stn* and *sop*B genes but *pef*A gene found only 1 sample (12.5%) . Combining between the phenotypic antimicrobial results and PCR pathotyping of different *Salmonella* spp. may be effective in providing a more accurate profile to understand the danger of spread of different virulence genes and multidrug resistance in *Salmonella* spp.

Key words: chickens, PCR, Salmonella enterica serovars.

Introduction

Salmonella enterica serovar *Typhimurium* and *Enteritidis* are the most frequently isolated serovars throughout the world leading to sever economic losses especially in poultry industry ***(Herikstad et al., 2002)***. Pathogensis of Salmonella is depending on many factors controlled by several genes that play the main role in virulence and

these genes are clustered on SPI ***(Murugkar et al., 2003).***

Antimicrobial-resistant strain of Salmonella spp. Are now wide spread all over the world causing of a great concern due to the spread of multi-drug resistant strains. In developed countries, The majorty of resistance strains are of zoonotic origin (it had become resistant in its animal host before transmitted to human host through food chain) ***(Molbak et al., 2002, Threlfall, 2002 and WHO, 2004).***

Integron means genetic elements that have the ability to integrate, by site specific re-combination, gene cassettes, which always confer antimicrobial resistance. 3 classes of integron have been known in detail and are involved in antimicrobial resistance.

Rowe et al. (2002) stated that the rapid spread of antimicrobial resistantance is incriminated to the presence of Class 1 integrons which found on the plasmid of Gram negatives bacteria.

There are many Salmonella pathogenicity islands, With SPI-1 and SPI-2 being the most important ones because they encode proteins of type III secretion system (TTSS) which is responsible for Salmonella success as an intracellular microorganism at different stages of invasion, intracellular replication and survival within the host ***(Marcus et al., 2000 and Hensel, 2004).***

Differences in virulence among Salmonella serovars and in the course of Salmonella infections in various host species have been attributed to the variable acquisition and evolvement of virulence genes. In serovar Typhimurium, at least 80 different virulence genes have been identified. A large part of these genes are clustered on the chromosome in distinct regions, called Salmonella pathogenicity islands (SPIs). At this time, five SPIs have been identified. In addition, several smaller clusters of virulence genes have been identified that are located in so-called pathogenicity islets ***(Marcus et al., 2000).*** The *inv*A gene has been recognized as an international standard for detection of Salmonella ***(Malorny et al., 2003).*** *hil*A gene which located in SPI1 is the key regulator of the SPI1 genes and type three secretion system. It is concluded that *hil*A is involved in long-term shedding and colonization of *S*. Enteritidis in the chickens caeca ***(Marcus et al., 2000)*** *inv*A and *hil*A are located on SPI-1 and are marker for Salmonella ***(Akbarmehr, 2010)***.

Additionally, an effector protein is the Salmonella outer protein B (*sop*B) that present in Salmonella pathogenicity islands -5 ***(Rahman, 2006).*** Many Salmonella serovars have large plasmids of different sizes that carry genes responsible for growth and survival within the host cell as *pef*A gene (plasmid encoded fimbriae) and *spv* gene (Salmonella plasmid virulence) ***(Gulig, 1990).*** Some virulence

genes are not presented on Salmonella pathogenicity islands, such as Salmonella enterotoxin (*stn*) the chromosomally-encoded ***(Prager et al., 1995 and Baumler et al., 1996)***.

An effector protein of the TTSS complex is *avr*A that contributes to the virulence of *Salmonella* spp. by limiting the host's inflammatory responses through the inducement of cell apoptosis, especially of macrophages, and by the inhibition of IL-8 and TNF-α ***(Collier-Hyames et al. 2002, Ben-Barak et al. 2006)***.

Fimbrial gene *bcf*C appeared widely distributed among Salmonella, these data are consistent with the essential functions of adhesion factors for the attachment and internalization processes that occur during pathogenesis ***(Borriello et al., 2012).***

Nowadays, antibiotic resistance of Salmonella had a received considerable care as the presence of multidrug-resistance (MDR) leading to failure of treatment ***(Yan et al., 2003)***. So, there is a great need for the development of methods for the rapid identification of Salmonella microorganism as a step of control.

The traditional methods for isolation and identification of *Salmonella* species by conventional culture methods take a long time, while polymerase chain reaction (PCR) gives the way to overcome these problems and promotes amplification and easily detection of the specific virulence genes of multidrug resistance Salmonella ***(Zhu et al., 1996).***

From the above**,** it is appeared better to provide valuable insights into the infection possibility and the estimation of virulence and antibiotic resistance in Salmonella strains from chickens to help in mapping out suitable preventive way to control the spread of this enteric microorganism in Egypt.

Aim of work: Isolation and identification of *Salmonella* Enterica from chicken, Serotyping of the recovered Salmonella isolates, Antibiogram for detection of multidrug resistant Salmonella isolates, Detection of class 1 integron in MDR isolates and its gene cassettes using RFLP technique.and detection of the most significant virulence genes for the obtained isolates by PCR.

Material and Methods

Bacterial strains:

Two Salmonella serotypes, Salmonella enterica serotype Typhimurium and Salmonella enterica serotype Lagos were isolated, biochemically and serologically identified previously testing their sensitivity to different antimicrobial drugs.

PCR screening of virulence genes and class 1 integron

A) DNA Extraction

DNA of different MDR Salmonella serotypes were extracted as described by boiling method ***(Rahn et al., 1992)***.

B) Purity assessment :

The concentration of DNA in µg/ml was measured at 260 and 280 nm by ultra-violet spectrophotometer (Shimadzu, Japan), then the ratio of reading at 260/280 was calculated. Pure DNA should have a ratio of > 1.8 as contamination with protein resulted in a significantly lower value.

PCR amplification and cycling protocol:

All PCR amplification reactions targeting the most important virulence genes of Salmonella serovars. As described in table (1)

C)* Agarose gel electrophoresis *(Sambrook et al., 1989):

An aliquot of each amplified PCR product (5ml) was electrophorsed on 15% agarose gel (Sigma) containing 0.5 mg/ml Ethidium bromide (Sigma) using 1X TBE buffer for 1 hour at100 V. The separated bands were visualized and photographed under an ultraviolet transillator. A 100bp ladder (Fermentas,Inc. Hanover, VSA) was used as a molecular size marker to determine the molecular weight of the PCR products.

Table (1): *Oligonucleotide primers sequences used for PCR amplification of somevirulence genes of salmonella.*

Target gene	Primer sequence 5 – 3	Amplified product(bp)	Reference
***inv*A**	GTGAAATTATCGCCACGTTCGGGCAA	284	Oliveira *et al*., (2003)
	TCATCGCACCGTCAAAGGAACC		
stn	TTG TGT CGC TAT CAC TGG CAA CC	617	Murugkar *et al*., (2003)
	ATT CGT AAC CCG CTC TCG TCC		
***pef*A**	TGT TTC CGG GCT TGT GCT	700	
	CAG GGC ATT TGC TGA TTC TTC C		
***sop*B**	tca gaa gRc gtc taa cca ctc	517	Huehn *et al*. (2010)
	tac cgt cct cat gca cac tc		
***bcf*C**	acc aga gac att gcc ttc c	467	
	ttc tgc tcg ccg cta ttc g		
***avr*A**	CCT GTA TTG TTG AGC GTC TGG	422	
	AGA AGA GCT TCG TTG AAT GTC C		
***hil*A**	CATGGCTGGTCAGTTGGAG	150	Yang *et al*., (2014)
	CGTAATTCATCGCCTAAACG		
Integron (hep 35 and hep 36)	TGCGGGTYAARGATBTKGATTT	491	White *et al*. (2000)
	CARCACATGCGTRTARAT		

Table (2): *PCR cycling programs and the amplicon sizes of Salmonella virulence genes and class 1 integron.*

Target gene	Specificity/location	Actual cycles	Final extension	Amplifid product size (bp)
***inv*A**	Comman virulence gene/SPI-1	35cycle 94˚C/30sec 55˚C/30sec 72˚C/30sec	72˚C/5 min	284
***hil*A**	Transcriptional/regulator/SPI-1	35cycle 94˚C/30sec 55˚C/30sec 72˚C/30sec	72˚C/5 min	150
***avr*A**	Salmonella induced inflammation/ SPI-1	35cycle 94˚C/30sec 55˚C/30sc 72˚C/30sec	72˚C/45 min	422
Stn	Enterotoxin/ chromosome	35cycle 94˚C/30sec 59˚C/30sec 72˚C/30sec	72˚C/10 min	617
***sop*B**	Effector protein/SPI-5	35cycle 94˚C/30sec 58˚C/30sec 72˚C/30sec	72˚C/10 min	517
pefA	Plasmid encoded fimbriae /plasmid	35cycle 94˚C/30sec 55˚C/30sec 72˚C/30sec	72˚C/10 min	700
***bcf*C**	Colonisation factor, fimbrial usher/ chromosome	35cycle 94˚C/30sec 53˚C/30ec 72˚C/30sec	72˚C/10 min	467
Integron	antimicrobial resistance/ Chromosome and plasmid	35cycle 94˚C/30sec 55˚C/30sec 72˚C/30sec	72˚C/10 min	491

NB: Initial Denaturation 94˚C/5 min

Results

PCR amplifications for some virulence genes of Salmonella isolates

PCR amplifications for some virulence genes in eight representative isolates revealed that all isolates were positive for *inv*A, *hil*A, *bcf*C, *stn*, *sop*B and *avr*A gene at 284, 150, 467, 617, 517 and 422 bp,but only one isolate was positive for *pef*A gene at 700bp, as revealed in Fig. (1).

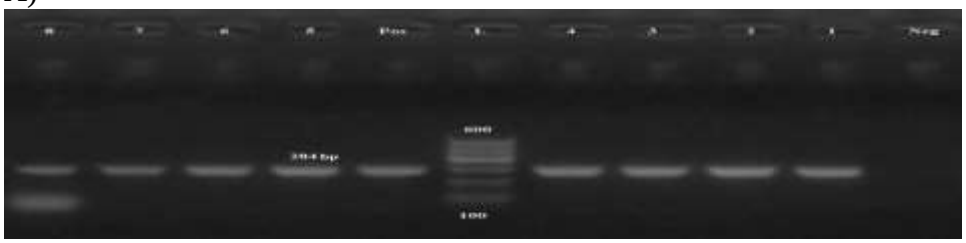
Detection of class 1 integron

Class 1 integron did not be detected among multidrug resistant tested isolates.

Finally, all serotypes analyzed were multidrug resistant and all of them carry different virulence genes simultaneously (*inv*A,*hil*A,*stn*,*sop*B,*avr*Aand *bcf*C) while only one strain contained

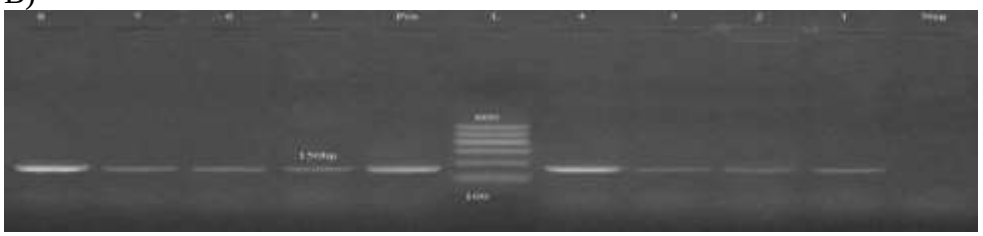
*pef*A gene. Additionally, antimicrobial resistance pattern was most prevalent to AMC, AM, CRO and SXT. As shown in Table (2).

A)



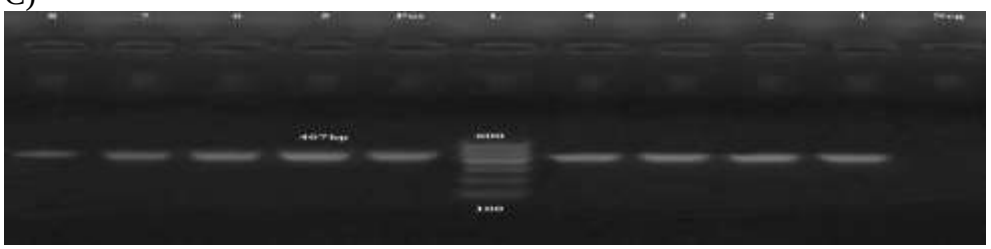
Amplified products of *inv*A gene at 284bp, Lane L : 100-600bp DNA Ladder, ,Lane Neg.: control negative ,Lane pos.: control positive,Lane 1 to 8: Positive bands for *inv*A gene , 1 and 2 (*Salmonella* Lagos) and 3-8 (*Salmonella* Typhimurium).

B)



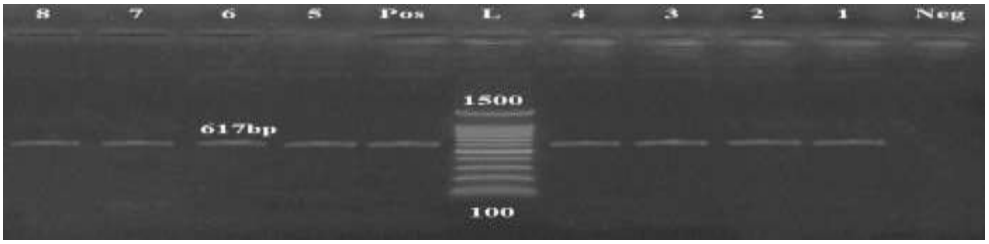
Amplified products of *hil*A gene at 150bp, Lane L : 100-600bp DNA Ladder.,Lane Neg.: control negative ,Lane pos.: control positive, Lane 1 to 8: Positive bands for *hil*A gene ,1 and 2 (*Salmonella* Lagos) and 3-8 (*Salmonella* Typhimurium).

C)



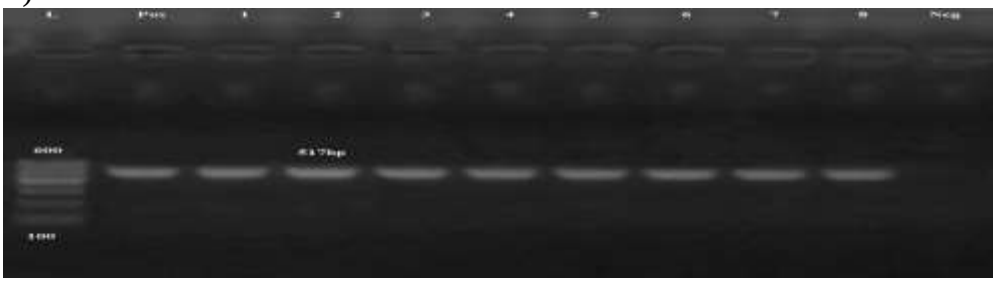
Amplified products of *bcf*C gene at 467bp, Lane L : 100-600bp DNA Ladder, Lane Neg.: control negative ,Lane pos.: control positive,Lane 1 to 8: Positive bands for *bcf*C gene , 1 and 2 (*Salmonella* Lagos) and 3-8 (*Salmonella* Typhimurium).

D)



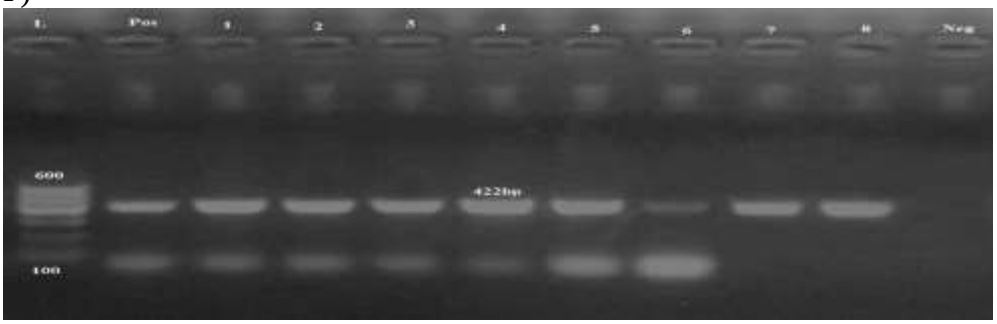
Amplified products of stn gene at 617bp, Lane L : 100-1500bp DNA Ladder Lane Neg.: control negative ,Lane pos.: control positive,Lane 1 to 8: Positive bands for stn gene , 1 and 2 (Salmonella Lagos) and 3-8 (Salmonella Typhimurium).

E)



Amplified products of sopB gene at 517bp, Lane L : 100-600bp DNA Ladder, Lane Neg.: control negative ,Lane pos.: control positive,Lane 1 to 8: Positive bands for sopB gene , 1 and 2 (Salmonella Lagos) and 3-8 (Salmonella Typhimurium).

F)



Amplified products of avrA gene at 422bp, Lane L : 100-600bp DNA Ladder. Lane Neg.: control negative ,Lane pos.: control positive, Lane 1 to 8: Positive bands for avrA gene ,1 and 2 (Salmonella Lagos) and 3-8 (Salmonella Typhimurium).

G)

Amplified products of pefA gene at 700bp, Lane L : 100-1000bp DNA Ladder, Lane Neg.: control negative ,Lane pos.: control positive,Only Lane 4: Positive bands for pefA gene of Salmonella Typhimurium.

Fig(1):*Agarose gel electrophoresis for amplified products of Salmonella virulence genes.*

Table (3) *Resistance pattern and the detected virulence genes among multidrug resistant Salmonella serovars***.**

Isolate code No.	Serotype	Resistance pattern	Number of Detected virulence gene
1	*S.* Lagos	AMC-AM-CRO-DO-SXT	*inv*A-*hil*A-*stn*-*sop*B-*avr*A-*bcf*C
2	*S.* Lagos	AMC-AM-CRO-DO-SXT	*inv*A-*hil*A-*stn*-*sop*B-*avr*A-*bcf*C
3	*S.* Typhimurium	AMC-AM-CRO-DO-CN	*inv*A-*hil*A-*stn*-*sop*B-*avr*A-*bcf*C
4	*S.* Typhimurium	AMC-AM-CRO-DO-SXT	*inv*A-*hil*A-*stn*-*sop*B-*avr*A-*bcf*C –*pef*A
5	*S.* Typhimurium	AMC-AM-CRO-DO-SXT	*inv*A-*hil*A-*stn*-*sop*B-*avr*A-*bcf*C
6	*S.* Typhimurium	AMC-AM-CRO-DO-SXT	*inv*A-*hil*A-*stn*-*sop*B-*avr*A-*bcf*C
7	*S.* Typhimurium	AMC-AM-CRO-DO-CN-AX	*inv*A-*hil*A-*stn*-*sop*B-*avr*A-*bcf*C
8	*S.* Typhimurium	AMC-AM-CRO-DO-C	*inv*A-*hil*A-*stn*-*sop*B-*avr*A-*bcf*C

N.B:* AMC,AM,CRO,DO and SXT were the most common pattern.
* *inv*A, *hil*A, *avr*A,*stn*, *bcf*C and *sop*B genes were all detected in all isolates and isolate code No. 4 has an additional *pef*A gene.

Discussion

Salmonellosis occurs worldwide in many countries and represent a major contributor to morbidities and mortalities with resultant economic costs in poultry ***(Antoine et al., 2008)***.

In general, molecular methods provide new insights into virulence and antimicrobial resistant genes of

Salmonella. They offer essential genetic information about the genes of interest, which compress one of the most practical and helpful aspects of PCR. Therefore, PCR is a rapid mehod for detection of Salmonella using PCR technique using the genus specific primer (*inv*A gene), which is a virulence determinant and play an important role in the Salmonella pathogenesis. ***(Darwin and Miller, 1999).*** As *inv*A gene encode a protein in the inner membrane of bacteria, which is necessary for invasion of the epithelial cells of the host, from the above results it is observed that amplification of *inv*A gene produced a PCR product 284 bp in all tested isolates of Salmonella (100%). These results confirmed the observations in Korea. ***(Hur et al., 2011)***, in Brazil ***(Chuanchuen et al., 2010)***, ***(Nde and Logue, 2008) (Olivera et al., 2002)***, ***(Lin et al., 2007)*** and ***(Malorny et al., 2003)*** that *inv*A gene is a useful marker for molecular detection of this pathogen by PCR ***(D'Souza et al., 2009 and Liang et al., 2011)***. Hyper invasive locus A gene (*hil*A gene) (transcription regulator), activiates the expression of *inv*A gene and is required to regulate TTSS. It was found in all tested isolates confirming results reported by ***(Pathmanathan et al., 2003)***,who detected the presence of the *hil*A gene in 33 Salmonella strains and 15 none of the Salmonella strains indicating 100% specificity for Salmonella *hil*A gene. Similar observations have been reported by other studies around the world in Iran ***(Amini et al., 2010)*** and in Brazil. ***(Campioni et al., 2012)***.

With respect to another chromosomally encoded *stn* virulence gene, a wide distribution of this gene had also been detected in all tested isolates. These results come in an agreement with another studies in India where *stn* gene was detected among different serotypes of Salmonella ***(Murugkar et al., 2003)*** and was detected in (99.3%) of the isolates ***(Zou et al., 2012)***.

Interstingly, some recognized protiens have some revelance to bacterial virulence as Salmonella pathogenicity island effector protiens. Here in, the isolates were screened for the *sop*B gene encoded by SPI-5 which was detected in all tested isolates of Salmonella serotypes. Typical results were reported in Egypt, ***(Kamelia et al., 2014)*** while was (99.3%) in North Carolina, ***(Zou et al., 2012)***, in Germany ***(Prager et al., 2000)*** and in UK, ***(Wood et al., 1998)*** stated that this gene is widely distributed. Plasmid encoded fimbriae (*pef*A gene) is located on plasmid was found in only one sample with (12.5%) . Different observation were reported for *pef*A gene as (17%) in North Dakota ***(Skyberg et al., 2006)***, (44.3%) in Ireland ***(Bolton et al., 2012)***, and 89% in India ***(Murugkar et al., 2003)***,when testing *bcf*C (bacterial colonization factor) the gene was

found in all tested isolates (100%) these finding exactly agrees with the results reported in Egypt, ***(Osman et al 2014)*** and ***(Kamelia et al., 2014)***.

The last examined virulence gene in this study was *avr*A gene that controls inflammation induced by Salmonella. In our study, *avr*A gene were detected in all Salmonella serotypes similar result were reported previously in England ***(Hopkins and Threlfall, 2004)***. The frequency variation could be caused by recombination that frequently occur in the location of this gene. These finding are important, since changes in the repertoire of proteins in *avr*A can changes in the ability of these serovars to adapt to new host ***(Parger et al., 2000).***

Up till now, more than 80 resistance gene, have been identified and carried on special structure called Class 1 integron which confer and represent the major consistent of multidrug resistance.(MDR). In the present study, Class 1 integron were not detected in all isolates. This could be attributed to similarities in the drug resistance phenotypes between integron positive and negative isolates indicate that MDR may or may not be integron related. This observation is similar to what has been reported in a previous study in the United States, class 1 integron was not always involved in the resistance of Salmonella isolates to antimicrobial agents ***(Mead et al., 1999 and Vugia et al., 2004)***.

Finally, the result shows that multidrug resistance strains are able to have many virulence genes which are important to stimulate pathogenicity of Salmonella. Which may increase the propensity of such such strain to be of a major clinical revalence, particularly if specific virulence factors are also carried.

Conclusion

Phenotypic antimicrobial results combining with PCR results for detection of virulence genes in different *Salmonella* spp. may be effective in providing a more accurate profile to understand the danger of spread of different virulence genes and multidrug resistance in *Salmonella* spp.

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الملخص العربي

التوصيف الجزيئي للأنماط المصلية للسالمونيلا انتريكا من مصادر مختلفة

يعتبر ميكروب السالمونيلا من الميكروبات الخطيرة نظرا لانه يعتبر احدي مسببات الامراض المشتركة التي تنتقل للانسان من الحيوان فله دور مهم في التسمم الغذائي في الانسان.
فغالبا ما تنتقل السالمونيلا للإنسان من خلال تناول الغذاء، فالدواجن هي واحدة من أهم الناقلات لعدوى السالمونيلا معتمدة في ذلك على العديد من عوامل اهمها هي جينات الضراوة.
في هذه الدراسة تم تجميع 270 عينة من الدواجن (الأرانب والبط ودجاج اللحم والكتاكيت) من مزارع اماكن مختلفة بمحافظة الشرقية.
اظهرت نتائج الاختبارات البيوكيميائية عزل 38 عترة من السالمونيلا بنسبة 14.07 %. اجريت الاختبارات المصلية لتصنيف عدد 8 عينات وهم يمثلوا جميع المزارع التي تم اخذ عينات منها وكانت النتائج 6 عزلات السالمونيلا التيفميوريم بنسبة 75٪ و 2 لسلالات لوجس السالمونيلا بنسبة 25٪.
اجريت الدراسة الجزيئية علي السلالات التي تم تصنيفها من السالمونيلا (6 سالمونيلا تيفيميوريم و 2 سالمونيلا لاجوس) متعددة المقاومة للمضادات الحيوية للكشف عن جينات الضراوة باستخدام تقنية PCR.
تم الكشف باستخدام ثمان انواع من جينات الضراوة المختلفة وهي *hil*A ,*inv*A, *avr*A , *bcf*C، *stn*, *sop*B و *pef*A والكشف عن الانتجرون وكانت النتيجة كالتالي:
أوضحت النتائج أن جميع العينات تحتوي علي *hil*A ,*inv*A, *avr*A , *bcf*C, *sop*B و*stn* بنسبة 100% بينما وجد *pef*A في عينة واحدة . ولم تحتوي اي من تلك العينات علي الانتجرون.