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DETECTION OF SOME VIRULENCE GENES IN SALMONELLA SPECIES ISOLATED FROM DUCKS AND DUCK EGGS

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ABSTRACT

Four hundred and fifty (450) samples were collected, 200 from internal organs (liver, caecum, gall bladder and spleen) of 50 ducks, 200 from internal organs of 50 ducklings and 50 unhatched duck eggs (dead in shell embryo) from different farms and hatcheries in Dakahlia Province. All samples were cultured on specific media for isolation and serological identification of *Salmonellae* spp. A total of 71 samples were positive to *Salmonella* with total percentage (15.8%). 29, 37 and 5 with (14.5%, 18.5% and 10%) positive samples were isolated from ducks, ducklings and duck eggs, respectively. Serotyping of the isolated *Salmonellae* from ducklings were five *S.infantis, four S.*kentucky, ten *S.typhimurium,* six S.newport, six *S.enteritidis* and six untypeable *Salmonella* with a percentage of (13.5%), (10.8%), (27 %), (16.2%), (16.2%) and (16.2%), respectively. Three *S.* derby, six *S.typhimurium*, five *S.*kentucky*, three S.* shubra*, four S.* enteritidis, three S.newport and five untypeable *Salmonella* were isolated from ducks with a percentage of (10.3%), (20.7%), (17.2%), (10.3%), (13.8%), (10.3%) and (17.2%), respectively. On the other hand only one of each *S.typhimurium*, S. enteritidis, S. newport, S. infantis and untypeable Salmonella were isolated from duck eggs with a percentage of (20%) for each one of these isolates. S.typhimurium was the most prevalence isolates *and showed high resistance to streptomycin (100%),* lincomycin (88%), erythromycin (82%), oxytetracycline (76%) and trimethoprim-sulfamethoxazole (70%). Moderate resistance to neomycin (59%), gentamycin (59%), amoxicillin (53%), ampicillin (53%) and chloramphenicol (47%) we reported. Meanwhile, the lowest resistances were against doxycycline (41%), flumequine (35%), norfloxacin (18%), ciprofloxxacin (6%) and colistin (6%). Conventional PCR assay detected virulence genes (*inv*A, *mgt*C, *sop*B and *bcf*C) using specific primer for each gene in all examined S.typhimurium samples. This study highlights that, there was significant association between *inv*A*, mgt*C*, sop*B and *bcf*C virulence genes and pathogenicity of *S.typhimurium* which showed high multiple antibiotic resistance which require strict regulations of the use of antibiotics in duck farms to minimize the resistant bacterial strains.

Key words: PCR, duck, virulence genes, S.typhimurium, antibiotic resistance.

INTRODUCTION

Salmonellosis is a zoonotic bacterial disease of national and international importance. The worldwide distribution of Salmonellosis often parallels the patterns of trade of animal products and food, and the migration patterns of human and animals (Gilbert *et al*., 2010).

Avian Salmonellosis is an important disease causing serious impediment to the development of poultry industry especially in developing countries of Asia and Africa. Since no "effective" immuno-prophylactic measures are available for the disease till now, strict biosecurity is the only alternative to preclude the disease (Rajagopal *et al*., 2013).

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The pathogenicity of *Salmonella* depends on a series of factors associated with the bacterium, the bird itself and the conditions in which the bird is raised. Attachment and penetration of the bacterium into digestive mucosa is a prerequisite for systemic infection (Rychlik *et al*., 1999). Polymerase chain reaction (PCR) is molecular biology technique which has taken up an increasingly significant space in the field of laboratory diagnostics, allowing the detection of various pathogens such as *Salmonella* species in different kinds of food. PCR can reduce the time required to detect and identify the agent with high specificity and sensitivity (Santos *et al.,* 2001).

Most bacterial pathogens including pathogenic *Salmonella* species have multiple virulence properties that enable them to invade and survive within the host and ultimately cause a disease (Marcus *et al*., 2000).

Detection and monitoring of drug-resistant *Salmonella* are important to substantiate the choice of antibiotics for the treatment of clinical Salmonellosis

and to assess the risk of exposure of multiple drug resistant strains (Yang *et al*., 2002).

This study was planned to estimate the prevalence of salmonella serovars implicated in the majority of infection in ducks and duck eggs and to determine some virulence genes associated with *S.* typhimurium infection in addition to study antimicrobial resistance profile that help to reduce the spread of resistant *salmonella* among the diseased ducks via planning a proper control program.

MATERIALS AND METHODS

Samples:
A total number of (450) samples from ducklings, ducks and duck eggs were collected as shown: (200) ducklings samples from internal organs (liver, caecum, gall bladder and spleen) of (50) ducklings, 1-3 weeks old (freshly dead, diseased living and apparently healthy birds), (200) ducks samples from internal organs (liver, caecum, gall bladder and spleen) of (50) ducks (freshly dead, diseased living and apparently healthy birds) and (50) unhatched duck eggs (dead in shell embryo). All samples were obtained from different duck farms and hatcheries located in Dakahlia Governorate under aseptic condition in ice box and transferred to the laboratory. Collected samples were cultured within a limit time.

Clinical and Postmortem examination:
All birds were examined clinically, then sacrificed and immersed in disinfectant before being autopsied. Gross pathological changes were recorded, summarized and presented with results for both freshly dead and clinically sick ducks.

Isolation of salmonellae:
The collected samples were transferred to test tubes containing Tryptose broth and Selenite F-broth and incubate at 37 ºC for 18-24 hours, followed by subculturing on blood agar, MacConkey's agar and Xylose lysine desoxycholate agar plates and incubated aerobically at 37 ºC for 18-24 hours *ISO* 6579 (2002).

Biochemical identification of the isolates:
The growing colonies on various plates were examined morphologically, culturally and biochemically (Indole, Nitrate Reduction, Voges Proskaur, Citrate Utilization, Urease, Sugar Fermentation and Coagulase test) Quinn *et al.* (2002).

Serological identification of salmonellae:
Serological identification of purified strains of Salmonella using available agglutinating Salmonella test sera (Denken Selken Co. LTD, Tokyo, Japan) according to Kauffmann (1973).

PCR technique for detection of virulence genes (Oliveira *et al*., 2003):
PCR test was carried out for seven S.typhimurium samples in PCR unit in Animal Health Research Institute, AHRI for detection of virulence genes. PCR amplification kit the QIAamp®DNA Mini Kit (Cat. No. 51304-Qiagen) was used according to manufacturer's instructions.

DNA extraction:
Chromosomal DNA extraction from samples was performed using the QIAamp DNA Mini kit (Qiagen, Germany, GmbH) with modifications from the manufacturer's recommendations. Briefly, 200 µl of the sample suspension was incubated with 10 µl of proteinase K and 200 µl of lysis buffer at 56 ºC for 10 min. 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 100µl of elution buffer provided in the kit.

Oligonucleotide Primer:
Primers used were supplied from Metabion (Germany) are listed in Table (1).

PCR amplification:
Primers were utilized in a 25- µl reaction containing 12.5 µl of Emerald Amp 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.

Analysis of the PCR Products:
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 was loaded in each gel slot. A gelpilot 100 bp DNA Ladder (Qiagen, Germany, GmbH) and generuler 100 bp ladder (Fermentas, Thermo) were used to determine the fragment sizes. The gel was photographed by a gel documentation system (Alpha Innotech, Biometra) and the data was analyzed through computer software.

Table 1: Primers sequences, target genes, amplicon sizes and cycling conditions.

Target gene	Primers sequences	Amplified segment (bp)	Primary denaturation	Amplification (35 cycles)			Final extension	Reference
				Secondary denaturation	Annealing	Extension		
invA	GTGAAATTATC GCCACGTT CGGGCAA TCATCGCA CCGTCAAAGG AACC	284	94˚C 5 min.	94˚C 30 sec.	55˚C 30 sec.	72˚C 30 sec.	72˚C 10 min.	**Oliveira *et al*., 2003**
sopB	TCA GAA GRCGTC TAACCA CTC TAC CGT CCT CAT GCA CAC TC	517	94˚C 5 min.	94˚C 30 sec.	58˚C 40 sec.	72˚C 45 sec.	72˚C 35 Sec.	**Huehn *et al*. 2010**
mgtC	TGA CTA TCA ATGCTC CAG TGA AT ATT TAC TGG CCGCTA TGC TGT TG	677						
bcfC	Acc Aga Gac Att Gcc Ttc C Ttc Tgc Tcg Ccg Cta Ttc G	467			53˚C 40 sec.			

In vitro antibiotic susceptibility test:
Seventeen *S.typhimurium* isolates were subjected to antibiotic sensitivity test against 15 commonly used antibiotics. The antimicrobial susceptibility profile against oxytetracycline, ampicillin, erythromycin, gentamycin, streptomycin, neomycin, lincomycin, chloramphenicol, ciprofloxacin, trimethoprim-sulfamethoxazole, amoxicillin, doxycycline, flumequine, colistin and norfloxacin were tested by disk diffusion methods according to Clinical and Laboratory Standards Institute (CLSI, 2012).

RESULTS

Clinical and postmortem findings of examined birds:
The clinical signs observed on examined ducks suffered from salmonellosis were lowered heads, closed eyes, drooping wings, ruffled feathers, Whitish watery diarrhea, death, pasted vent, conjunctivitis, retarded growth and lameness.

The postmortem examination of both freshly dead and sacrificed ducks suffered from salmonellosis were unabsorbed yolk sac (ducklings), congested internal organs (Liver, kidney and spleen) with enlarged gall bladder, pinpoint necrotic foci on the bronzed liver, spleenomegaly with congested kidneys and white cecal core with enteritis.

Table 2: The prevalence of *Salmonella* species recovered from collected samples.

samples	Number of examined samples	Results			
		Number of positive samples	%	Number of negative samples	%
Ducks (internal organs)	200	29	14.5	171	85.5
Duckling (internal organs)	200	37	18.5	163	81.5
Duck eggs	50	5	10	45	90
Total	450	71	15.8	379	84.2

Table 3: Serotyping of the isolated *Salmonella* species**.**

Isolated strains	Duckling, No. and (%)	Duck, No. and (%)	Duck eggs, No. and (%)
S. Kentucky	4 (10.8%)	5(17.2%)	0 (0 %)
S. Derby	0 (0%)	3(10.3%)	0 (0%)
S. Typhimurium	10(27%)	6(20.7%)	1 (%20)
S. Newport	6 (16.2%)	3 (%10.3)	1 (%20)
S. Enteritidis	6 (16.2%)	4(13.8%)	1 (%20)
S. Infantis	5 (13.5%)	0 (%0)	1(20%)
S. Shubra	0 (0%)	3 (%10.3)	0(0%)
Untyped salmonella	6 (16.2%)	5 (17.2%)	1 (%20)
Total	37(100%)	29 (100%)	5(100%)

S. typhimurium was the most prevalence isolates

Table 4: Antibiotic sensitivity and resistance pattern for (17) S.typhimurium isolates.

Antibiotic	Antimicrobial class	Sensitive		Resistant	
		No.	(%)	No.	(%)
Streptomycin	Aminoglycosides	0	0	17	100
Lincomycin	Lincosamides	2	12	15	88
Erythromycin	Macrolydes	3	18	14	82
Oxytetracycline	Tetracycline	4	24	13	76
Trimethoprim-sulfamethoxazole	Sulphonamides	5	30	12	70
Gentamycin	Aminoglycosides	7	41	10	59
Neomycin	Aminoglycosides	7	41	10	59
Ampicillin	B- Lactam	8	47	9	53
Amoxicillin	B -Lactam	8	47	9	53
Chloramphenicol	Phenicols	9	53	8	47
Doxycycline	Tetracyclines	10	59	7	41
Flumequine	1st Generation Quinolones	11	65	6	35
Norfloxacin	2 nd Generation Quinolones	14	82	3	18
Colistin	Peptides	16	94	1	6
Ciprofloxacin	2 nd Generation Quinolones	16	94	1	6

Table 5: Result of PCR assay for detection of virulence genes of S. *typhimurium.*

Sample	**Results**			
	invA	**mgtC**	**sopB**	**bcfC**
1	+	+	+	+
2	+	+	+	+
3	+	+	+	+
4	+	+	+	+
5	+	+	+	+
6	+	+	+	+
7	+	+	+	+

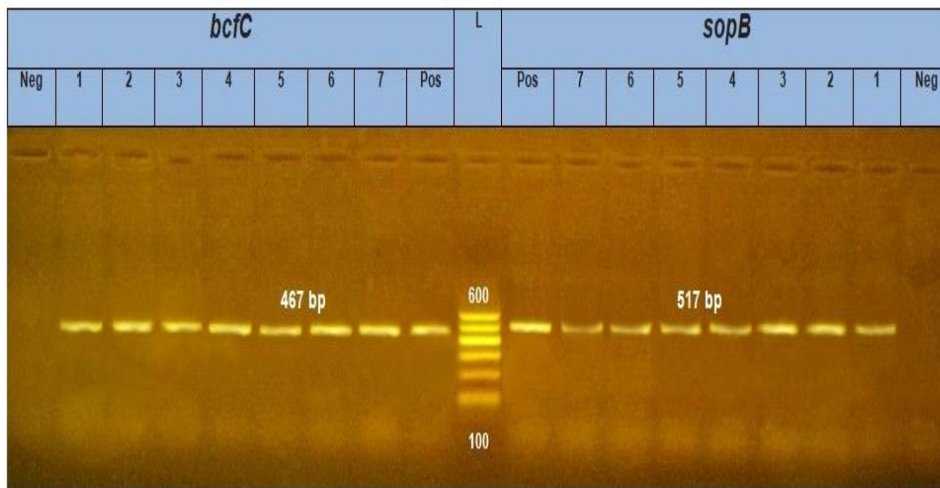

Figure (1): Agarose gel electrophoresis showing amplification of 467bp fragment using bcfc primer and 517 bp fragment using sopB primer.
L: 100- 600 bp ladder.
Lane (1-7): positive samples.
Neg: negative
Pos: positive

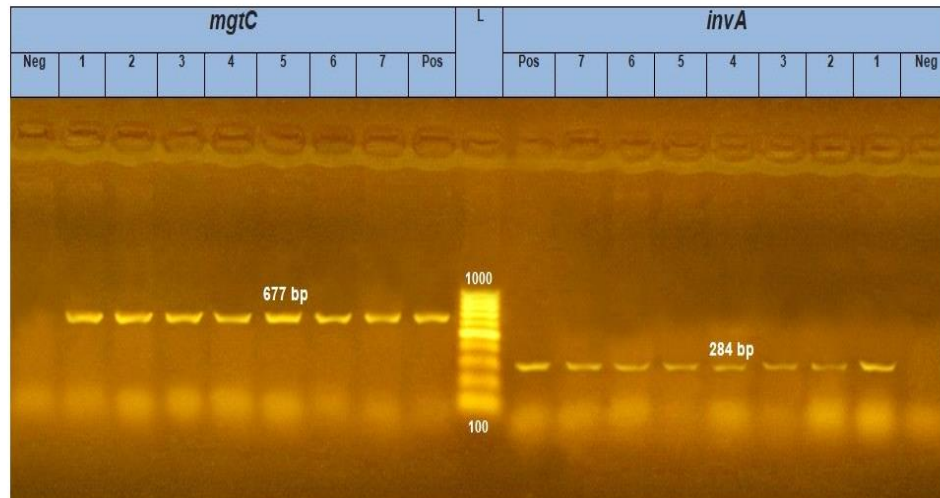

Figure (2): Agarose gel electrophoresis showing amplification of 677bp fragment using mgtC primer and 284 bp fragment using invA primer.
L: 100- 1000 bp ladder.
Lane (1-7): positive samples.
Neg: negative
Pos: positive

DISCUSSION

Salmonella is one of the most important pathogen that can infect poultry and causing serious illness in human through consumption of contaminated food products. Egypt has experienced the repeated introduction and dissemination of a wide range of *Salmonella* serotypes that have become a major concern for both human and animal health. Detection of Salmonellosis is considered a good tool in protection of poultry industry FAO (1994). Further more, *Salmonella* infection is one of the most important bacterial diseases in poultry causing heavy economic loss through mortality and reduced production Haider *et al*. (2004).

The present investigation revealed that the clinical signs of salmonellosis in ducks were lowered heads, closed eyes, drooping wings, ruffled feathers, whitish watery diarrhea, death, pasted vent, lameness and conjunctivitis. These findings are in agreement with those observed by Riberio *et al*. (2005).

Postmortem lesions of salmonellosis recorded in ducks were unabsorbed yolk sac, congested internal organs, pinpoint necrotic foci on the liver with bronze discoloration, spleenomegaly with congested kidneys

and white cecal core. These findings are similar with those observed by Saif *et al*. (2008).

In our study 71 out of 450 samples from ducklings, ducks and duck eggs were found to be positive to *Salmonella* (15.8 %) as shown in table (2). This result was in agreement with that of Lam *et al*. (2002) and Nayera (2012) who reported (12.7%) and (14.42%), respectivily. Meanwhile, Asawy and Abd El-latif (2010) reported (3.3%) and Abd El-Tawab *et al*. (2015) reported (9.6%). Twenty nine samples from ducks were found to be positive with a percentage of (14.5 %). This finding was in accordance with that of Mondai *et al.* (2008) who recorded (13.07%). On the other hand El-Zeedy *et al*. (2007) and Tasi and Hsing (2005) recorded (7.8%) and (4.6%) for Salmonella, respectivily.

In the current study 37 ducklings samples were found positive for Salmonella isolation at a percentage of (18.5 %). This result was in the same direction with Huang *et al.* (1994) who reported (19.3%) and Lam *et al*. (2002) (18%). This result disagreed with Osman *et al*. (2014) who reported (12%). The prevalecce of isolation of Salmonella in ducklings (18.5%) was higher than that in ducks (14.5%). This was in accordance with that of Lam *et al.* (2002) and Tasi and Hsing (2005).

In we present study five duck eggs were positive for Salmonella isolation at rate of (10 %). This result agreed more or less with the results reported by *Shareef et al*. (1997) and Mohammed (2014) who reported a prevalence rate of (8%) and (9.09%), respectively. In the current study the isolated Salmonella serotypes from ducklings were *S.kentuckey, S.infantis,* S.typhimurium*, S.* newport and *S.enteritidis as* shown in Table (3) These findings were in agreement with that reported by *El-Zeedy et al*. (2007) who isolated *S.enteritidis, S.typhimurium* and *S.infantis* from ducklings. Meanwhile the isolated Salmonella serotypes from ducks were *S.derby, S.newport S.typhimurium, S.kentucky, S.enteritidis* and *S.shubra*. Similar results for serological identification were reported by Tasi and Hsing (2005) who isolated *S.typhimurium, S.newport and S.derby* from ducks. The isolated Salmonella from duck eggs were *S.typhimurium, S.infants, S.enteritidis* and *S. newport*. Mohammed (2014) was able to isolated *S.typhimurium* from duck eggs.

In the present study S. *typhimurium* was the most prevalence isolates. This might run parallel with Mituniewicz *et al*. (2007). But Hamad (2017) reported that S.enteritidis was the most prevalent isolates from ducks samples.

Regarding antibiotic sensitivity our study showed that S.typhimurium was highly resistant to streptomycin, lincomycin, erythromycin, oxytetracycline and trimethoprim-sulfamethoxazole in percentage of 100%, 88%, 82%, 76% and 70%, respectively as shown in table (4). This result nearly agreed with that recorded by Yoshida *et al*. (1993) who cited that Salmonella was resistant to oxytetracycline, streptomycin and trimethoprim-sulfamethoxazole and Oja and Adetosoye (2009) who reported that, S.typhimurium was resistant to erythromycin. In the present study S.typhimurium showed moderate resistance to neomycin (59%), gentamycin (59%), amoxicillin (53%), ampicillin (53%) and chloramphenicol (47%). This is in agreement with Mondai *et al*. (2008) and *Boris et al*. (2012). The highest sensetivity rates were (59%, 65%, 82%, 94% and 94%) for doxycycline, flumequine, norfloxacin, ciprofloxacin and colistin, respectively. This was in the same direction with Chashni *et al.* (2009) who reported that the sensitivity rates of S.typhimurium were to flumequine (74%) and norfloxacin (93%), Abd El-Hamid *et al. (*2003) norfloxacin (86.96%) and Nagappa *et al*. (2007) showed that colistin was highly effective aganist S.typhimurium. On the other hand Cardoso *et al*. (2006) reported that S.typhimurium was resistant to colistin.

Amplification of *inv*A gene now has been recognized as an international standard for detection of *Salmonella* genus Malorny *et al*. (2004). The *inv*A gene encodes a protein in the inner membrane of bacteria, which is necessary for invasion to the epithelial cells of the host Darwin and Miller (1999). Development of a PCR system remains a suitable molecular tool to diagnose *Salmonella* on the basis of *inv*A amplification and this has been reported by Bisi-Johnson *et al*. (2011).

In the present study, PCR assay was carried out for the detection of the *inv*A gene from the isolated strains. The result revealed that the gene was present in all of the isolates (100%) that was demonstrated by the presence of 284 bp PCR amplified fragment which agreed with Dione *et al*. (2011).

Concerning *bcf*C gene (fimberial gene) code for an outer membrane usher protein, which carried in the microbial genome as part of a transposable element allowing a better bacterial protein secretion during the intestine invasion and present in all strains with (100%).This result agreed with that of Nayera (2012) who present it in almost strains (95.7%). Regarding *sop*B gene encodes an inositol phosphate phosphatase that is transported into epithelial cells by the SPI-1 secretion system and indirectly affects chloride secretion by inositol phosphate signaling pathways. PCR assay was carried out for the detection of the *sop*B gene from the isolated strains. The result revealed that the gene was present in all of the isolates (100%). This was in accordance with that of Mohammed (2014). With respect to *mgt*C gene is required for intra-macrophage survival and growth in magnesium-depleted medium and *also* is essential for organisms that invade host cells as cited by *Alix* and

Blanc-Potard (2008), the *mgt*C gene was present in all examined isolates (100%). This result was similar to that of Zou *et al.* (2012). In the same regard Nayera (2012) and Mohammed (2014) were able to find mgtC gene in (54.3%) and (53.3%) of examined strains, respectivily.

The current investigation concluded that, there was significant association between *inv*A, *mgt*C, *sop*B and *bcf*C virulence genes and pathogenicity of *S.typhimurium* which expresed high multiple antibiotic resistance which require strict regulations of using antibiotics in duck farms to minimize the resistant bacterial stranis.

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الكشف عن بعض جينات الضراوة للسالمونيلا المعزولة من البط وبيض البط

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أجريت هذة الدراسه على عدد ٤٥٠ عينة من الاعضاء الداخلية (كبد و طحال و حوصلة مرارية و اعورين) لعدد ٥٠ بطة بالغة و٥٠ بطة صغيرة عمر من أسبوع إلى ثلاثة اسابيع (سليم ظاهريا ومريض ونافق حديثا) وكذلك عدد٥٠ بيضة كابس (بداخلها جنين ميت) تم تجميعها من مزارع بط ومعامل تفريخ مختلفة فى محافظة الدقهلية. وبإجراء الفحص الظاهرى للطيور المصابة ظاهريا تبين وجود إسهالات مائية بيضاء وخمول وضعف عام وعرج مع تبلل وإتساخ حول فتحة المجمع. وبإجراء الصفة التشريحية تبين وجود تضخم في الكبد والطحال والحوصلة المرارية مع ظهور اللون البرونزى للكبد وأنزفة نقطية على جدار الامعاء وكذلك وجود مواد متجبنة فى الأعورين وعلامات إلتهاب الصرة فى الاعمار الصغيرة. أظهرت نتائج الفحص البكتريولوجى أن اجمالى عدد العينات الايجابية للسالمونيلا ٧١ بنسبة عامة ١٥,٨% وكان عدد العينات الإيجابية فى البط البالغ ٢٩ بنسبة ١٤,٥ % و فى البط الصغير ٣٧ بنسبة ١٨,٥ % أما فى بيض البط كانت ٥ عينات بنسبة ١٠ %. تم تصنيف المعزولات سيرولوجيا إلى ٦عترات سالمونيلا تيفيميوريم و٥ كنتاكى و٤ إنتريتيدس و٣ عترات لكلا من سالمونيلا ديربى و نيوبورت و شوبرا من البط البالغ بينما صُنف ١٠عترات تيفيموريم و٦ نيوبورت و ٦ إنتريتيدس و ٥ إنفانتس و٤ كنتاكى من عينات البط الصغير وكانت معزولات بيض البط معزولة واحدة لكلا من سالمونيلا تيفيميوريم و إنتريتيدس وإنفانتس ونيوبورت بنسبة ٢٠% لكل معزولة. سجلت السالمونيلا تيفيميوريم أعلى نسبة عزل لذلك. تم إجراء اختبار تفاعل انزيم البلمره المتسلسل لعدد سبع معزولات من السالمونيلا تيفيميوريم لتحديد والكشف عن بعض جينات الضراوة invA , SopB) bcfC, magC) وتبين وجودهم جميعا فى كل المعزولات الممرضة. وبإجراء إختبار الحساسيه لعدد ١٧ معزوله للسالمونيلا تيفيميوريم لقياس نسبة مقاومتها لعدد (١٥) مضادا حيويا شائع إستخدامها في مزارع البط. وقد تبين أن نسبة المقاومه كانت ١٠٠% للاستربتومايسين و ٨٨% للينكومايسين و ٨٢% للإرثرومايسن و ٧٦% للاوكسيتتراسيكلين و ٧٠% للترايميثوبريم سلفاميثاذول و٥٩% للنيومايسين و ٥٩% للجنتاميسين و ٥٣ % للاموكيسلين و ٥٣% للامبيسلين و ٤٧% للكلورمفينيكول و ٤١% للدوكسى سيكلين و ٣٥% للفليموكوين بينما كانت اقل نسب مقاومة ١٨% للنورفلوكساسين ٦% لكلا من السيبروفلوكساسين والكولستين. ولقد ألقت هذة الدراسة الضوء على إرتباط وجود جينات (magC, invA, SopB, bcfC) مع قوة وضراوة المعزولات الممرضة للسالمونيلا تيفيميوريم. ونظرا لارتفاع نسب مقاومة ميكروب السالمونيلا تيفيميوريم لأغلب المضادات الحيويه توصي هذه الدراسه بالحد من استخدام المضادات الحيويه بطرق غير علميه في مزارع البط واجراء الإختبارات المعمليه المناسبه لاختيار المضادات الحيويه الفعاله لتفادى ظهورعترات بكتيرية مقاومة للمضادات الحيوية .