Molecular Analysis of Some Virulence Genes of Salmonellae Isolated from Chicken at Sharkia Governorate

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Abstract

Poultry is one of the most reservoirs of Salmonella that can be transmitted to humans through the food chain causing high risk bacterial food poisoning. In the present study, bacteriological examination of 200 samples from broiler internal organs (liver,2ceci, un absorbed yolk sac) from1 day old chicks suffering from omphalitis and respiratory disorders after hatching, revealed 26 Salmonella isolates 13%. Serotyping of 26 Salmonella isolates by slide agglutination test using specific monovalent and polyvalent O and H Salmonella sera revealed eleven different Salmonella serotypes, with *S*. Enteritidis as the most prevalent serotype (38.5%) followed by *S*. Tamale (23.07%). , *S*. Typhymurium (19.2%), *S*. Kentucky (15.4%)and ,*S*.Heldberg (3.8%).most *Salmonella* isolates were sensitive to Ciprofloxacin ,sulfa methoxasole – trimethoprime , Chlorumphenicol and gentamycin .while they were resistant to Erythromycin ,Rifamycin , Amoxycillin /clavulinic acid ,colistin sulfate and cefotaxime . PCR detection of 6verulant genes of 5 strains of Salmonella isolates which proved to play an important role in the virulence of Salmonella in chicken. In this study detection of (invA gene, hilA gene, stn gene, spvc gene, fmH gene and pefA gene) is with percentages of (100%, 80%, 100%, 0%, 100% and 40%)respectively. From examined 5MDR Salmonella isolates, Integron class1were detected in all of them (100%) but no strain was found to harbor Integron class2.

Key words: Salmonella, virulence,genes,integrin,chicken .

Introduction

Salmonella isolates are considered as the most circulating and frequent bacterial agents causing disease in poultry and other avian specieS.It is associated with high mortality,morbidity and impaired production ***Sedeik et al. (2019).*** Salmonellosis has been associated with infection of broiler flocks that has the ability of vertical transmission to progeny ***Irshad et al. (2013).***

The high prevalence of multidrug resistant *Salmonella* to be commonly used antimicrobial in veterinary and public health sectors has emerged as a global problem that resulting in treatment failure ***(Piddock 2002, Molla et al.,2003 and Yan et al., 2003 Khemtong and Chaunchuen 2008)***.

The present work is planned out as an attempt to throw some spotlights on:

1. Isolation of *Salmonella* serovars from different poultry farms.
2. Biochemical and serological identification of *Salmonella* isolates.
3. Antimicrobial sensitivity test on *Salmonella* isolates.
4. Genotypic characterization of obtained *Salmonella* isolates to detect the most predominant virulence genes.
5. Detection of class1 and cass2 integrin in multi-drug resistant isolates by polymerase chain reaction

Materials and Methods

1. sampling

200 samples from broiler internal organs (liver,2ceci, un absorbed yolk sac) from1 day old chicks suffering from omphalitis and respiratory disorders after hatching. samples were collected aseptically to prevent cross contamination by wearing disposable gloves and using sterile sampling materials (swabs, bags and syringes). The samples were collected and transported in ice boxes with ice packs as early as possible to the laboratory for bacteriological examination and keeping on refrigerator.

2 Isolation of *Salmonella, biochemical* identification and serotyping

We follow ISO 6579 (2002) procedure. Suspected Salmonella colonies were confirmed serologically by Kauffman – White scheme (Kauffman, 1974) for the determination of (O) and (H) antigens using Salmonella antiserum (DENKA SEIKEN Co., Japan). and biochemically by (TSI) test, Urea hydrolysis test, Lysine decarboxylation test, Indole production test and Simmon Citrate utilization test. The isolates were then serotyped by the Animal health research institute in Dokki -Giza. Only

confirmed Salmonella were tested for their susceptibility to different antibiotic and the presence of the virulant gnes class1 and class2 integron.

3 Resistance to the antimicrobial agents

We detect susceptibility of isolates to antimicrobial agents following the Clinical and Laboratory Standards Institute ***(Clinical and Laboratory Standards Institute, CLSI, 2007)*** for the disk diffusion technique. The antimicrobials and concentrations tested were Amoxicillin/clavulanic acid, Norfloxacin, Gentamicin, Cefotaxim, Sulfamethoxazole/ trimethoprim, Ciprofloxacin, Nalidixic acid, Doxycyclinem, Erythromycin, Chloramphenicol, Colistin sulphate,Rifamycin The inhibition zones were scored as sensitive, inter- mediate susceptibility or resistant according to the CLSI .

4 **Polymerase chain reaction for amplification of the most important virulent genes of Salmonella isolates** (invA gene, hilA gene, stn gene, spvc gene, fmH gene and pefA gene)

a.Extraction of DNA according to QIAamp DNA mini kit instructions.

b. Preparation of PCR Master Mix according to Emerald Amp GT PCR mastermix (Takara) Code No. RR310A kit.

Results&Discussion

Results revealed 26 Salmonella isolates from200 samples with percentage (13%). Serotyping of 26 Salmonella isolates by slide agglutination test using using specific monovalent and polyvalent O and H Salmonella sera detected eleven different Salmonella serotypes, with S. Enteritidis as the most prevalent serotype (38.5%) followed by S.Tamale (23.07%). , S. Typhymurium (19.2%), S. Kentucky (15.4%)and ,S.Heldberg (3.8%).most Salmonella isolates were sensitive to Ciprofloxacin ,sulfa methoxasole – trimethoprime , Chlorumphenicol and gentamycin .while they were resistant to Erythromycin ,Rifamycin , Amoxycillin /clavulinic acid ,colistin sulfate and cefotaxime .

Examination of samples collected from diseased and died saso chicks from different hacharies in Sharkia using SMT,26 *Salmonella* isolates was isolated in an over prevalence of 13%(26/200. The results of serological identification in present study detected 5 different serovars from 26 *Salmonella* isolates, *S*.Entertides predominated with higher percentage (38.5%)while the remained isolates were serotyped as.

S.Tamale, *S*.Typhymurium, *S*.Kentucky, *S*.Heldberg,with percentages (23.07%),

(19.2%) ,(15.4%),(3.8%), repectively.

Genotypic characterization of some *Salmonella* serotypes:

S*.*Enteritides **(**105),*S*.Tamale(13),*S*.Typhimurium(17),*S*.Kentucky and *S*.Heldberg (97) were subjected to PCR genotyping for detection of some virulent genes(*inv* A, *fimH*,*hil*a,*pef*,and*spv*C)and Integron(Int1 and Int2) .

It was evident that the oligonucleotide primer pairs targeting the genes under study successfully amplified the DNA extracted from tested *Salmonella* isolates for each primer.

As expected, PCR confirmation of bacteriology positive strains was documented by appearance of amplified DNA fragment of 284bp for the invA gene,a target for *Salmonella* genus in all examined serotypes(100%)

Detection of class 1 integron and class 2 integron among multidrug resistant isolates:

From examined 5MDR Salmonella isolates, Integron class1were detected in all of them (100%) but no strain was found to harbor Integron class2(0%) as shown in figures 7&8.

Association between phynotypic antimicrobial results and genotypic detection of some virulence genes of different *Salmonella* species could be effective in providing a more accurate profile for understanding the dangerous spread of virulence genotypes and antibiotics resistance in *Salmonella* serovars.

From the above mentioned results, it is important to note that amplification of invA gene as *Salmonella* species specific and virullance factor in this study produced a PCR product of approximately 284bp in all *Salmonella* species tested100%). All 5 *Salmonella* serotypes which Identified phenotypically were found to possess invAgene indicating that PCR result from *invA*gene detection in agreement with these of conventional methods.

Several authors proposed a rapid, sensitive and specific PCR method using primers for *invA* gene for the detection of *Salmonella* serotypes in may clinical samples within a maximum of 12hr, thus confirming its affiliation to *Salmonella* species ***(Lampel et al.,2000; ferretti et al.,2001; schneder et al 2002 and Ammar et al.,2014).***

In addition, ***oliveria et al. (2002) and Malorny et al. (2003***) and ***Lin et al. (2007)*** reported that *inv*Agene abled to identify all eamined *Salmonella* serovar by PCR technique.

In the last decade, there has been a wide interest in the use of PCR technique. PCR have been

applied to detect different species of several microorganisms, to differentiate closely related species ***Settani and Corsetti, (2007)***. The primary advantages of PCR tests are increased sensitivity and less time required to process samples in the laboratory when compared to standard culture method ***Lampel et al. (2000).***

In the present work, PCR approaches have been applied to dtect different virulent genes that are (invA,hilA, pefA,stn,spvc and fimH) and also detect integron class 1 and integron class 2 that is responsible for antibiotic resistance .

Finally, amplification 0f *invA* and *hliA* genes now has been recognized as an international standard for detection of genus *Salmonella* ***Maloeny et al. (2003).***

It is alarming that *Salmonella* induced diarrhea is a complex phenomenon involving several pathogenic mechanisms including production of enterotoxin which is mediated by *stn* gene. Therefore, a uniplex PCR assay was carried out for detection of this gene in the representative *Salmonella* isolates. The results revealed the presence of stn gene in all tested *Salmonella* isolates with a PCR product at 619bp. This finding is in a greement with earlier reports of ***Parger et al. (1995) and Morogkar et al. (2003)*** who detected *stn* gene among different Discussion 78 Serovars of *Salmonella*, indicating that *stn* gene is widely distributed among *Salmonella* irrespective of their Serovars and source of isolation.

Moreover, fimbriae play an important role in the pathogenicity of bacteria which is a key factor for bacteria invasion and survival inside the host cells ***Finaly and falkow (1989).*** *PefA* gene is encoded by pef operon located in a plasmid ***Friedrich et al. (1993).***

Indeed, among the isolates analysed in this study *pefA* gene was present in 40%of these isolates. This result is go hand in hand with Mona 2014 that dectect *pefA* with percentage 41.1%. On the contrary this result is markedly lower than that presented *by* ***Wood Ward et al. (1996)*** who found that 97.9% of *S.*Entertidis isolates was positive for the presence of this gene and ***Morugkar et al. (2003)*** who found pefa in 89% of different *Salmonella* serovars in India.

Furthermore, *Salmonella* is plasmid virulence (spvc) is also related to survival and growth of the bacterium in host cells ***Swamy et al. (1996).***

In this study, no isolate of *Salmonella* was positive for the presence of spvc gene that is differ from than that observed by ***Amini et al. (2010)*** who detecyed spv in 30%of

S.enteritidis strains isolated from poultry and ***Ihab et al. (2013)*** who found that the incidence of spvc genes was 31.5%in *S*.Enteritidis and 30%in *S* .Typhymurium isolates from poultry.

Salmonella enterica serovar Enteritidis is presently the major causative agent of food born gastro enteritis in human worldwide and an important source of bacterial infection on poultry farms resulting in public health problems and economic loss .Infection by *S*. Enteritidis is initiated by the attachment and colonization of gut mucosa ,which seem to be essential stage in the pathogenesis of salmonellosis .Increasing Discussion 78 evidence suggests that bacterial fimbriae play an important role in these processes ***Dagmara (2006).***

S.Enteritidis genome contains many putativefimbrial operons :afg,bcf,fim and pef ***Edward et al. (2002).***

Studies on *S*.Typhymurium revealed that FimH adhesion is responsible for bacteria binding to intestinal epithelial cells ***Thankavel et al. (1999).***

The *fim* H gene produced an amplified PCR product at164bpin all *Salmonella* tested.

S. enterica serovar Typhymurium express type 1fimbriae that enable the bacteria to bind to eukaryotic cell and have been implicated in mediation.

We have previously shown that *fim*H mutants of *Salmonella* are unable to adhere to eukaryotic cell, demonstration that FimH is critical for the ability of type 1 fimbriae to specifically bind to host cells ***Brett et al. (2011).***

Association between phynotypic antimicrobial results and genotypic detection of some virulence genes of different *Salmonella* species could be effective in providing a more accurate profile for understanding the danderous spread of virulence genotypes and antibiotics resistance in *Salmonella* serovars. Overall, multidrug resistant *Salmonella* serotypes were also cabable for exhibiting several virulence determinants which are very important to induce *Salmonella* pathogenicity. This result corresponds closely to a previous report published by ***Amminin et al. (2011)*** who found the virulence gene invA, hila located Discussion 77 on SPI-1 and other virulence genes not present on it such as stn and spvc were detectable in all multidrug resistant *Salmonella* isolates.

It is important to note that *Salmonella* can easily acquire multiple drug resistance to most antimicrobial and transform them to human through food chain.

Recently, the generation and the transformation mechanism of the drug resistance gene have become a hot research topic in order to control the spread of multi drug resistance bacteria.

Integron, a noval DNA element, mediating the integration of antibiotic resistance gene through site specific recombination has great impact on human health. The back bone structure of an integron is probably the most prevalent type of integrin harboured by such isoltes ***Sunde.2005 and Ren et al. (2013).***

In the present study, class1 integron and class 2 integron, was screened among 5 multidrug resistance *Salmonella* strains using specific primer for integrase (IntI1) and (IntI2) gene The result revealed the presence of class1 integrin in all tested serotypes and absence of class 2 integrin in all tested serotypes. Other reports have also revealed the prevalence of class 1 integron in gram negative isolates from food samples as 41.3% in united states ***Khaista et al. (2008)*** ,45% in china ***Li et al.(b2011)***, 49 % in Australia ***White et al. (2001)*** 99% In Bortugal ***Antunes et al. (2004)*** Indicating that class 1 integron are wide spread among gm - ve isolates.

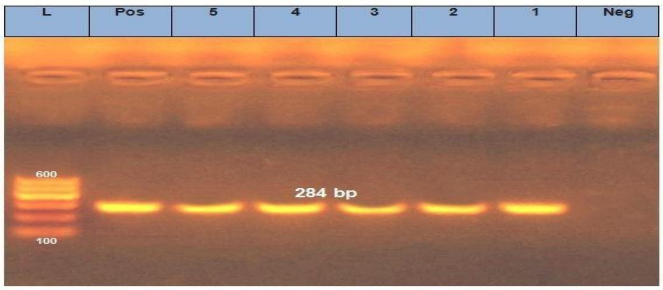

Fig (1): Agarose gel electrophoresis showing the result of PCR for detection of invAgene from 5*Salmonella* strains

Lanes 1,2,3,4,5: *Salmonella* species

Lane Pos: positive *invA* control.

Lanes 1,2,3,4,5: positive amplification of 284bp for *invA* gene of different *Salmonella* species.

Lane L: the DNA molecular weight marker (Gelpilot 100bp ladder).

Lane Neg: negative *invA* control. (control negative).

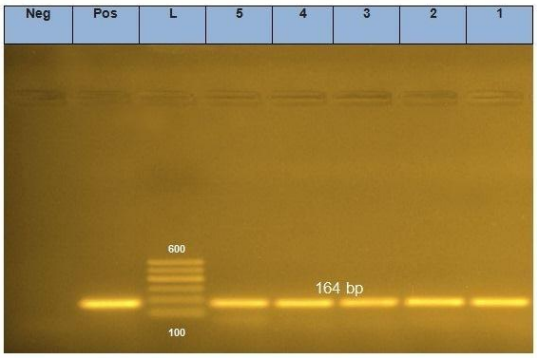

Fig. (2): Agarose gel electrophoresis showing the result of PCR for detection of *fim* H gene from5 *Salmonella* strains
Lanes 1,2,3,4,5: *Salmonella* species
Lane Pos: positive *fimH* control.
Lanes 1,2 ,3,4 and5: positive amplification of 164bp for *fimH* gene of different *Salmonella* species.
Lane L: the DNA molecular weight marker (Gelpilot 100bp ladder).
Lane Neg: negative *fimH* control. (control negative).

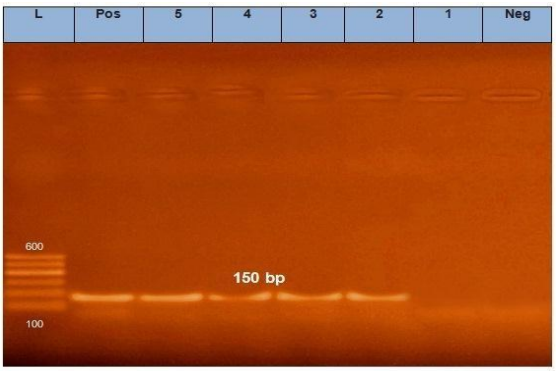

Fig. (3): Agarose gel electrophoresis showing the result of PCR for detection of *hil* A gene from5 *Salmonella* strains
Lanes 1,2,3,4,5: *Salmonella* species
Lane Pos: positive *hil A* control.
Lanes 2 ,3,4 and5: positive amplification of 150bp for *hil A* gene of different *Salmonella* species.
Lane 1: negative amplification of 150bp for *hil A* gene of different *Salmonella* species.
Lane L: the DNA molecular weight marker (Gelpilot 100bp ladder).
Lane Neg: negative *hil A* control. (control negative).

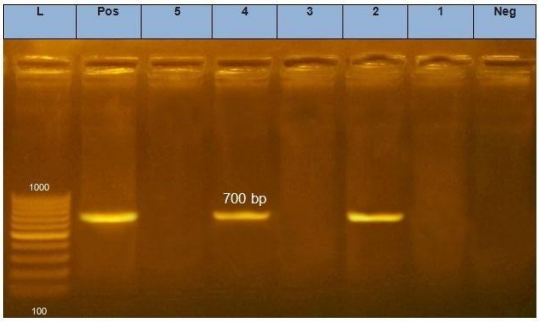

Fig. (4): Agarose gel electrophoresis showing the result of PCR for detection of pef gene from5 *Salmonella* strains

Lanes 1,2,3,4,5: *Salmonella* species

Lane Pos: positive *Pef* control.

Lanes 2 and4: positive amplification of 700bp for *Pef* gene of different *Salmonella* species.

Lanes 1,3 and5: negative amplification of 700bp for *Pef* gene of different *Salmonella* species.

Lane L: the DNA molecular weight marker (Gelpilot 100bp ladder)

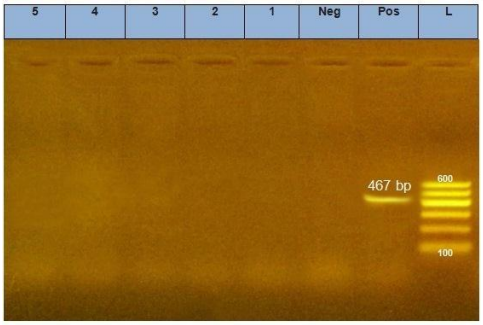

Fig. (5): Agarose gel electrophoresis showing the result of PCR for detection of *spv*C gene from5 *Salmonella* strains

Lanes 1,2,3,4,4,5 *Salmonella* species

Lane Pos: positive *spvC* control (refrence strain)**.**

Lanes 1,2,3,4, 5: negative amplification of 467bp for *spvC* gene of different *Salmonella* species.

Lane L: the DNA molecular weight marker (Gelpilot 100bp ladder).

Lane Neg: negative *spvC* control. (control negative)

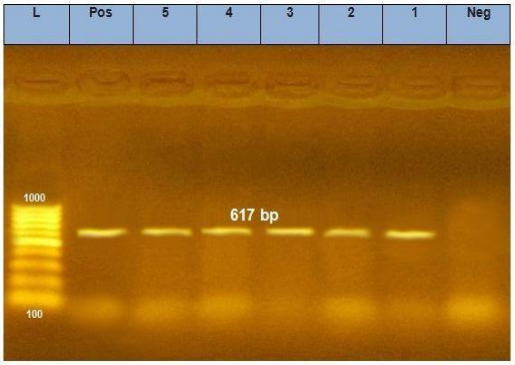

Fig. (6): Agarose gel electrophoresis showing the result of PCR for detection of stn gene from5 *Salmonella* strains

Lanes 1,2,3,4,4,5 *Salmonella* species

Lane Pos: positive *stn* control (refrence strain

Lanes 1,2,3,4,5: positive amplification of 617bp for *qnrS* gene of different *Salmonella* species.

Lane L: the DNA molecular weight marker (Gelpilot 100bp ladder).

Lane Neg: negative *stn* control. (control negative).

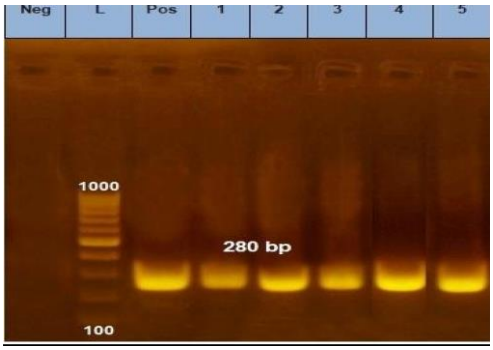

Fig. (7): Agarose gel electrophoresis showing the result of PCR for detection of *Int1* from5 *Salmonella* strains

Lanes 1,2,3,4,4,5 *Salmonella* species

Lane Pos: positive ***Int1*** (refrence strain

Lanes 1,2,3,4,5: positive amplification of 280bp for ***Int1*** of different *Salmonella* species.

Lane L: the DNA molecular weight marker (Gelpilot 100bp ladder).

Lane Neg: negative ***Int1*** control. (control negative).

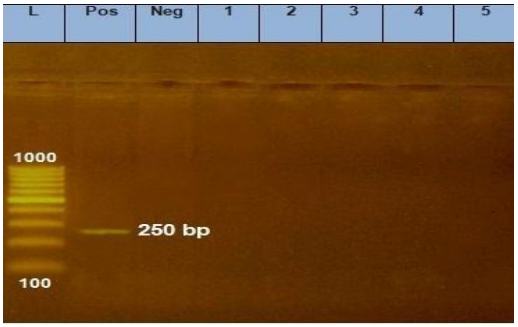

Fig. (8): Agarose gel electrophoresis showing the result of PCR for detection of *Int2*from5 *Salmonella* strains
Lanes 1,2,3,4,4,5 *Salmonella*species
Lane Pos: positive ***Int2*** control (refrence strain)**.**
Lanes 1,2,3,4, 5: negative amplification of 250bp ***Int2*** of different *Salmonella* species.
Lane L: the DNA molecular weight marker (Gelpilot 100bp ladder).
Lane Neg: negative ***Int2*** control. (control negative).
Based on the phenotypic and genotypic characterization,the isolates were categorized in five different well defined profiles as shown in table()

Table (1): *Relationship between drug resistance and presence of virulent genes, Int 1 and Int 2 among Salmonella isolates*:

Code No.	Sample origin	Antimicrobial sensitivity (R&I)	Virulent genes	*Int 1*	*Int 2*
105 *S*.E	Liver	AMC-S-NA-CTX- SXT--E-C-CT-RF.	*InvA-stn- fimH*.	+	-
13 *S*.Ta	Liver	CTX-E-CT-RF	*invA-hilA-stn pefA-fimH*.	+	-
17 *S*.Ty	Yalk Sac	S-CN-NA- CTX- DO-E-C-CT-RF.	*invA-hilA-stn fimH*.	+	-
33 *S*.K	Liver	AMC-NA-CTX- SXT-DO-E-C-CT-RF.	*invA-hilA-stn pefA-fimH*.	+	–
97 *S*.H	2ceci	AMC-CN-NA-CTX- E-CT-RF.	*invA-hilA-stn fimH*.	+	-

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الملخص العربي

التحليل الجزيئي لبعض جينات الضراوه المعزوله من الدجاج في محافظة الشرقيه

الدواجن هي واحدة من أكثر مصادر السالمونيلا التي يمكن أن تنتقل إلى الإنسان من خلال السلسلة الغذائية مسببة مخاطر تسمم غذائي جرثومي عالي الخطورة. في هذه الدراسة ، أظهر الفحص البكتريولوجي لـ 200 عينة من أعضاء الداخليةمن كتاكيت عمرها يوم واحد تعاني من التهاب السرة واضطرابات في الجهاز التنفسي بعد الفقس ، أظهر 26 عزلة من السالمونيلا بنسبة13٪. أظهر التنميط المصلي لـ 26 عزلة من السالمونيلا عن طريق اختبار تراص الشرائح باستخدام مصل السالمونيلا أحادي التكافؤ ومتعدد التكافؤ وحامض السالمونيلا 11 نمطًا مصليًا مختلفًا من السالمونيلا ، مع S. Enteritidis باعتباره النمط المصلي الأكثر انتشارًا (38.5٪) يليه S. تامالي (23.07٪). ،س. تيفيموريوم (19.2٪) ، S. Kentucky (15.4٪ ،) S.Heldberg (3.8٪) معظم عزلات السالمونيلا كانت حساسة لسيبروفلوكساسين ، سلفا ميثوكساسول - تريميثوبريم ، كلورومفينيكول وجنتامايسين بينما كانت مقاومة للإريثروميسين ، ريفاميسين ، أموكسيسيللين. / حمض الكلافولينيك وكبريتات الكوليستين وسيفوتاكسيم. كشف تفاعل البوليميراز المتسلسل (PCR) لـ 6 جينات فائضة لخمس سلالات من عزلات السالمونيلا والتي ثبت أنها تلعب دورًا مهمًا في ضراوة السالمونيلا في الدجاج. في هذه الدراسة تم الكشف عن (جين InvA ، جين hilA ، جين stn ، جين spvc ، جين fmH وجين pefA) بنسب (100٪ ، 80٪ ، 100٪ ، 0٪ ، 100٪ و 40٪) على التوالي. من عزلات السالمونيلا MDR5 التي تم فحصها ، تم اكتشاف فئة 1 Integron في كل منهم (100٪) ولكن لم يتم العثور على سلالة تأوي Integron class2.

كلمات البحث: السالمونيلا ، جينات الضراوه، الإنتجرون ، الدجاج.