Prevalence and characterization of Salmonella species isolated from broilers

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Abstract
This study was conducted to determine the prevalence of *Salmonellae* in broilers farms in Dakahlia Governorate, Egypt. A total of 1000 samples that collected from 200 broiler chickens (40 apparently healthy, 80 diseased chickens and 80 freshly dead broiler chickens).These samples included liver, caecum, heart blood, spleen & kidney. The colonial morphology, microscpical and biochemical identifications of the isolates revealed the presence of 37 *Salmonella* isolates out of 200 chickens (18.5%) representing: 3 from apparently healthy chicken (7.5%), 21 from diseased chickens (26.25%) and 13 from freshly dead broiler chickens (16.25%).The rate of recovery of *Salmonellae* from the different internal organs showed that high recovery rate was from liver, caecum, spleen, heart then kidney as the follow (9.5%), (5.5%), (4.5%), (3%) and (2%), respectively. The serotyping of the isolated *salmonellae* from chickens were eight *S. enteritidis*, one *S. maccles Field*, two *S. wingrove*, one *S. eingedi*, three *S. rissen*, two *S. derby*, two *S. vejle*, one *S. magherafelt*, two *S. berta*, two *S. enterica sub.spp salamae*, one *S. gueuletapee*, one *S. blegdam*, five *S. kentucky*, two *S. newport*, two *S. agona* and two *S. virchow*. Gentamycin, ciprofloxacin, colistin sulphate and enrofloxacin were found to be the most effective antimicrobials drugs while erythromycin and flumequine were the most resistant antibiotic against the isolates. PCR assay was carried out for six serovars (*S. enteritidis, S. maccles Field, S. rissen, S. derby, S. magherafelt* and *S. enterica sub.spp salamae*) to detect the presence of *invA*, *sopB* and *stn* genes. All serovars had the three genes.
Keywords: Salmonella spp., Broilers, Prevalence, characterization

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. Salmonellosis is an important health problem and a major challenge worldwide. *Salmonella spp.* are recognized as the most causative agents of food poisoning. These organisms are Gram negative and rod shape which have been divided into over 2700

serotypes based on somatic, flagellar and capsular antigens ***(Gallegos et al, 2008).*** *Salmonellae* are short bacilli, 0.7-1.5 x 2.5 µm, Gram-negative, aerobic or facultative anaerobic, positive catalase, negative oxidase; they ferment sugars with gas production, produce H2S, are non sporogenic, and are normally motile with peritricheal flagella, except for *Salmonella Pullorum* and *Salmonella Gallinarum*, which are nonmotile ***(Forshell and Wierup, 2006).***

The genus *Salmonella* is divided into two species *Salmonella enterica* and *Salmonella bongori*; *Salmonella enterica* itself is comprised of 6 subspecies. They are *S. enterica subsp. enterica, S. enterica subsp. arizonae, S. enterica subsp. diarizonae, S. enterica subsp. indica, S. enterica subsp. houtenae or I, II, IIIa, IIIb, IV and VI*, respectively ***(Popoff and Minor, 1997).***

Salmonella enterica serovar typhimurium and S. enterica serovar enteritidis are the most frequent isolated serovars worldwide ***(Chiu et al, 2010).*** In Egypt *S. enteritidis* were isolated from broiler chicken, chicken meat and food poisoning patient. The clinical illness characterized by fever, nausea and diarrhea, vomition and abdominal pain after an incubation period of 12 to 72 hrs ***(Ammar et al, 2010).***

Many of the virulence genes of *S. enterica* are chromosomal genes located on pathogenicity islands referred to as Salmonella Pathogenicity Islands (SPI). These genes are believed to have been acquired by Salmonella from other bacterial species through horizontal gene transfer. They responsible for host cell invasion and intracellular pathogenesis. Other virulence factors of *Salmonella* include production of endotoxins and exotoxins, and presence of fimbrie and flagella ***(van Asten & van Dijk, 2005)***.

This study was planned to identify biochemically and serologically the prevalent *Salmonella* species in broilers farms in Dakahlia Governorate, Egypt. Also, for detection of common virulence genes of Salmonella using Polymerase Chain Reaction.

Material and methods

Sample collection

A total of 200 samples from broilers farms were collected for Salmonella isolation and these samples include liver, caecum, spleen, heart and kidney. All samples were put in sterile plastic bags in ice box and transported directly to Mansoura laboratory (Animal Health Research Institute).

Isolation of Salmonella according to ISO 6579 (2002) method

Each sample was inoculated separately in selenite F broth and incubated at 37°C for not more than 18 hours or Rappaport-Vassiliadis Soya broth (RVS) and incubated at 42°C for 24 hours. Then a loopful

from selective enriched media was streaked onto plates of MacConkey's, Salmonella–Shigella (S.S) and xylose lysine deoxycholate and incubated overnight at 37 °C. Typical colonies were picked and further tested by standard biochemical methods and serotyped using specific commercial sera according to the Kauffmann–White scheme **(Kauffmann, 1974)**.

Identification of Salmonella isolates:

Microscopic examination

Films from suspected purified colonies were prepared, fixed and stained with Gram's according to ***Quinn et al (2002)*** then examined microscopically

Biochemical Identification according to ISO 6579 (2002) method:

Purified isolates were examined by different biochemical reactions either by oxidase, urea hydrolysis, H_2S production on TSI, lysine decarboxylation, indole, methyl red test, Voges-Proskauer, citrate utilization, motility test and Analytical profile index 20 E (API 20 E)

Serological identification:

The preliminarily identified isolates biochemically as *Salmonella* were subjected to serological identification according to Kauffman-White Scheme **(Kauffman, 1974)** for determination of somatic (O) and flagellar (H) antigens using slide agglutination test.

Detection of common virulence genes in Salmonella isolates using PCR:

1. Extraction of DNA ***(Oliveira et al, 2003).***

2. Preparation of PCR Master Mix according to Emerald Amp GT PCR mastermix (Takara).

3. Cycling conditions of the primers during cPCR.

4. DNA Molecular weight marker.

5. Agarose gel electrophoreses ***(Sambrook et al, 1989).***

Antibiotic sensitivity testing according to ISO 6579 (2002) method:

Determination of the susceptibility of the isolated strains to antibiotic discs was adopted using the disc diffusion technique according to ***Finegold and Martin (1982).***

Results

The results illustrated in Table 1 demonstrated the prevalence of *Salmonella spp.* in examined chickens.

Bacteriological examination of samples allover seasons of the year revealed that salmonella was recovered in 37 samples with an incidence rate 18.5% (37 out of 200) as shown in Table 1.

The recovery rate of *Salmonella* from internal organs is clarified in Table 2.

As shown in Table 2, a high level of Salmonella infection was found in liver (9.5%) followed by caecum (5.5%); spleen (4.5%); heart (3%) and kidney (2%).

All *Salmonella* suspected isolates showed smooth red coloured colonies with black center on XLD while on Hektone enteric it appeared as deep blue colonies but on MacConkey's agar appeared as pale, colorless smooth, transparent and raised colonies and on Salmonella Shigella (S-S) agar, Salmonella produce colourless colonies with black centers due to H2S production. The staining characters appeared as Gram negative, non-spore forming & short rod shaped. Biochemically, all *Salmonella* suspected isolates were non-lactose fermenting colonies and negative oxidase, urea hydrolysis, indole and Voges-Proskauer tests. Meanwhile, most isolates produced H_2S and positive methyl red, citrate utilization and lysine decarboxylation.

The results of serotyping of isolated *Salmonella* species were observed in Table 3. The isolated salmonella (37) were serotyped using "O" and "H" antisera to determine the salmonella serotypes as eight *S. enteritidis*, one *S. macclesfield*, two *S. wingrove*, one *S. eingedi*, three *S. rissen*,two *S. derby*, two *S. vejle*, one *S. magherafelt*, two *S. berta*, two *S. enterica sub.spp salamae*, one *S. gueuletapee*, one *S. blegdam*, five *S. kentucky*, two *S. newport*, two *S. agona*, two *S. virchow* were isolated from broilers with percentage of (21.62%), (2.7%), (5.4%), (2.7%), (8.1%), (5.4%), (5.4%), (2.7%), (5.4%), (5.4%), (2.7%), (2.7%), (13.5%), (5.4%), (5.4%) and (5.4%) respectively.

Six *salmonella* serotypes (*S. enteritidis, S. macclesfield, S. rissen, S. derby, S. Magherafelt and S. enterica sub.spp salamae*) examined for detection of virulence genes as invA, stn and sopB by conventional PCR. All examined serotypes have the three genes as demonstrated in photos 1, 2 &3.

All *Salmonella* isolates were tested for antibiotic sensitivity test to 10 different antibiotics. Gentamycin, ciprofloxacin, colistin sulphate and enrofloxacin were the most effective (100% effectivity of each) followed by florphenicol (93.75%), neomycin (81.25%). Meanwhile, erythromycin and flumequine were the most resistant antibiotic against the isolates (87.5%). Also, resistance to doxycycline hydrochloride was (81.25%) and ampicillin was (75%).

Table (1) *Incidence of Salmonella infection in examined chickens*

Examined chicken	Number of examined chicken	Number of positive	%
Apparently healthy chicken	40	3	7.5
Diseased chicken	80	21	26.25
Freshly dead chicken	80	13	16.25
Total	200	37	18.5

Table (2) *Rate of recovery of Salmonella from internal organs.*

Examined organs in 200 chicken	Number of positive	Percentage of positive
Liver	19	9.5
Caecum	11	5.5
Spleen	9	4.5
Heart	6	3
Kidney	4	2
Total	49	24.5

Table (3) *Serotyping of isolated Salmonella species*

Type of isolated Salmonella strains	**Antigenic analysis**	**Number of positive chicken**	**Percentage of positive (%)**
Salmonella enteritidis	O: 1,9,12.H 1 g, m, H2	8	21.62
Salmonella macclesfield	O: 9,46.H1 g, m, S, H2 1,2,7.	1	2.7
Salmonella Wingrove	O: 6,8. H1 C , H2 1,2	2	5.4
Salmonella eingedi	O: 6,7. H1 F,g,t, H2 1,2,7	1	2.7
Salmonella rissen	O: 6,7,14. H1 f,g. H2 -	3	8.1
Salmonella derby	O: 1,4,[5],12 .H1 F, g. H2[1,2]	2	5.4
Salmonella Vejle	O: 3,[10],[15].H1 e, h, H2 1,2	2	5.4
Salmonella magherafelt	O: 8,20. H1 I, H2 1,w	1	2.7
Salmonella berta	O: 1,9,12.H1 [F],g, [t] H2 -	2	5.4
Salmonella enterica sub.spp salamae	O: 1,4,[5],12.H1 F,g,t. H2 Z6	2	5.4
Salmonella gueuletapee	O:9,12, H1 g,m,s,H2 __	1	2.7
Salmonella blegdam	O:9,12, H1 g,m,q,H2 __	1	2.7
Salmonella kentucky	O: 8,20. H1: i, H2: Z6	5	13.5
Salmonella newport	O :6,8,20. H1 :e,h , H2 :1,2	2	5.4
Salmonella agona	O:1,4(5),12.H1:f,g,s, H2: (1,2)	2	5.4
Salmonella virchow	O:6,7,14. H1: r, H2: 1,2	2	5.4

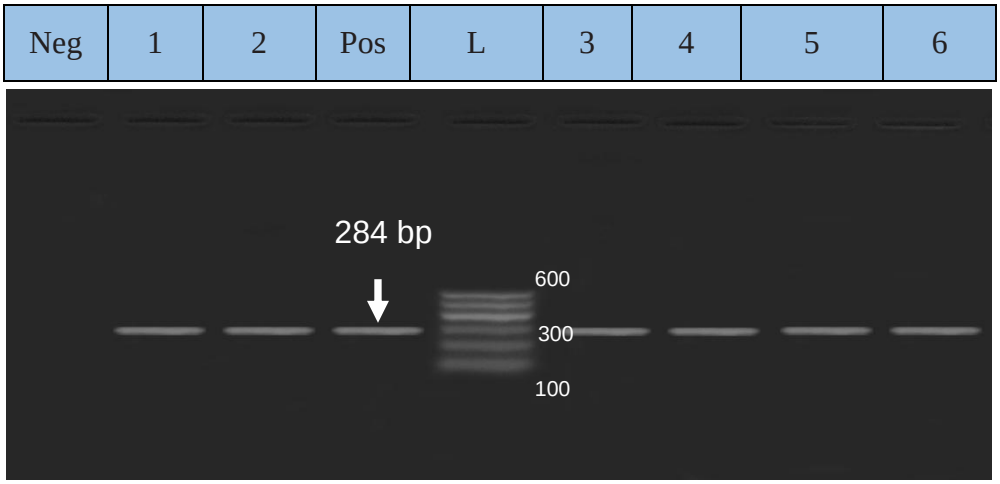

Photo (1): Agarose gel electrophoresis showing *Salmonella* specific PCR of *Salmonella* isolates using primer set for the *inv*A (284 bp) gene. Lane L: 100-600pb DNA ladder; Pos.: Positive control; Neg.: Negative control; Lane 1, 2,3,4,5 &6 examined *Salmonella.*

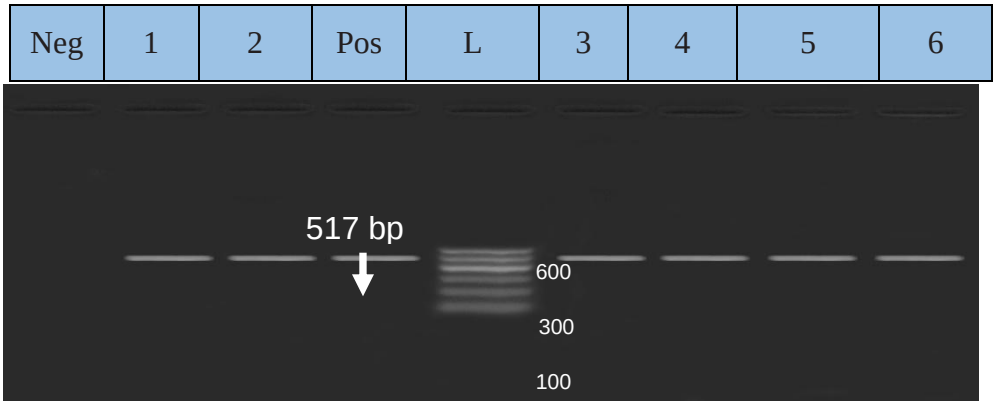

Photo (2): Agarose gel electrophoresis showing *Salmonella* specific PCR of *Salmonella* isolates using primer set for the *sop*B gene (517 bp). Lane L: 100-600pb DNA ladder; Pos.: Positive control; Neg.: Negative control; Lane 12,3,4,5 &6 examined *Salmonella.*

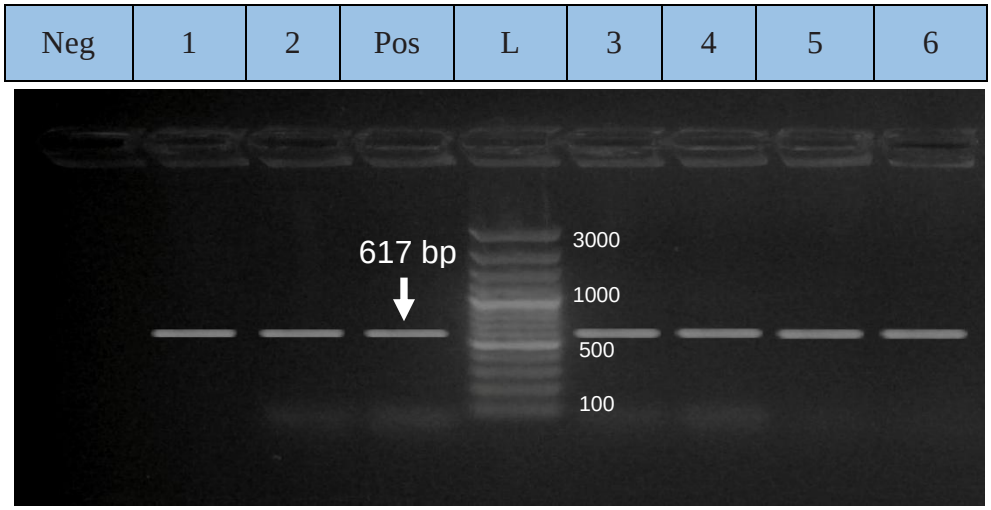

Photo (3): Agarose gel electrophoresis showing *Salmonella* specific PCR of *Salmonella* isolates using primer set for the *stn* (617 bp) gene. Lane L: 100-3000pb DNA ladder; Pos.: Positive control; Neg.: Negative control; Lane 1, 2,3,4,5 &6 examined *Salmonella.*

Discussion

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)***. In the present study, the incidence of *Salmonella* in broilers was 18.5% (37 out of 200 chickens) and these results agree with ***Kudaka et al (2006)*** who found that 18% of broilers were positive for *salmonella*. Also, ***EFSA (2007)*** reported that *Salmonella spp*. present with 20.3% in the broiler flocks in the European Union and **Kaushik *et al.*, (2014)** isolated *Salmonella* from chicken meat with 23.7%. On the other hand, lower incidence was recorded by ***Hassan et al (2003)*** (5.51%) and ***Abd El-Ghany et al (2012)*** (4.48%). However, ***Bada-Alambedji et al (2006)*** reported that *Salmonella* present in (62.5%) in examined chickens with higher incidence. The difference in the prevalence rates may be due to socio-economic factors.

Recovery of *Salmonella* species from internal organs of the examined chickens were higher from liver followed by caecum, spleen, heart and kidney 9.5%, 5.5%, 4.5%, 3% and 2% respectively. It was clear from these results, showed higher isolation rate of *Salmonella* species from liver and this similar to ***Chaiba et al (2009)*** isolated a higher level of *Salmonella* from liver (11.11 %). However, ***Cox et al (2007)*** isolated higher level of *Salmonella* from spleens followed by liver and ceca

of 6 weeks old broilers with 15%,10% and 8% respectively while in 8 weeks old broilers, were 51%, 48% and 65% of the livers, spleens, and ceca, respectively. But, ***Selvaraj et al (2010)*** found that the higher percentage of *Salmonella spp*. were isolated from chicken meat (8.00%) followed by liver and spleen (6.25% each), intestine and intestinal contents (5.26%), kidney and gall bladder (3.57%).

Serological identification of isolated *Salmonella* species revealed higher incidence of *S. enteritidis* (21.62%) followed by *S. kentucky*(13.5%), *S. rissen* (8.1%), 5.4% for each *S. wingrove, S. derby, S. vejle, S. berta, S. enterica sub.spp salamae, S. newport, S. agona & S. virchow* and 2.7% for each *S. maccles field, S. eingedi, S. magherafelt, S. gueuletapee & S. blegdam.*These results agree with that reported by ***Nagwa et al (2012); Dahal (2007); Kanashiro et al (2005); Shah and Korejo (2012); Putturu et al (2012) and Abd El-Ghany et al (2012).*** They recorded that the predominant serotypes of *Salmonella* was *S. enteritidis*. In contrast, ***Kaushik et al (2014)*** isolated *S. enteritidis* with 0.4% and *S. newport* with 2.6%. Moreover, ***Roy et al (2002)*** isolated *S. Kentucky* and *Salmonella enteritidis* with percentage of 21.64% and 5.15%, respectively.

Oliveira et al (2003) revealed that PCR method is high specificity and sensitivity and more importantly a less time-consuming procedure than standard microbiological techniques for detection and identification of Salmonella. PCR assay using the invA primers specific for Salmonella spp. considerably decreases the number of false-negative results which commonly occur in diagnostic laboratories. Amplification of invA is now recognized as an international standard procedure for detection of *Salmonella* genus. In this study, PCR assay was carried out for the detection of the *inv*A gene from six isolated strains (*S. enteritidis, S. macclesfield, S. rissen, S. derby, S. magherafelt and S. enterica sub.spp salamae*) has revealed that the gene was present in all of the isolates (100%) that was demonstrated by the presence of a 284 bp PCR amplified fragment. The results obtained in the present study were in corroboration with ***Malmarugan et al (2011); Nagappa et al (2007) and Dione 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%) which was demonstrated by the presence of a 517 bp PCR product. The results obtained in the present study were in corroboration with ***Eckmann et al (1997)***. Also, PCR assay carried out for the detection of the stn gene in *Salmonella* isolates has revealed that the gene was present in all the isolates (100%) that was demonstrated by the presence of a 617 bp PCR product. These findings are in

agreement with ***Murugkar et al (2003); Prager et al (1995) and Rahman H. (1999)***. Observations from the present study indicated that the stn gene is widely distributed among the *Salmonella* serovars.

In this study all *Salmonella* strains were sensitive to gentamycin, ciprofloxacin, colistin sulphate and enrofloxacin and this agree with ***Ramachandranpillai and Mangattumuruppel (2013)*** who reported that all the strains were sensitive to at least four antibiotics as gentamicin, chloramphenicol, ceftriaxone and ciprofloxacin. But on the contrary ***Yah and Eghafona (2007)*** reported that the isolates were highly resistant to ampicillin, chloramphenicol, gentamycin and tetracycline and this agree with the present study as all examined *salmonellae* were resistant to ampicillin except *S. enteritidis, S. derby, S. agona and S. wingrove*. ***Abd El-Rahman et al (2000)*** reported that *salmonella* species were sensitive to enrofloxacin and this agrees with the present study.

It could be concluded that there are high level of *Salmonella* isolation in broilers evaluated in this study may be attributed to horizontal and/or vertical transmission of *Salmonella* to the chicks. Also, the high rates of antibiotics resistance found in the present study can be explained by the abuse of antibiotics agents given to poultry in Egypt as prophylaxis, growth promoters or treatment. The multiple resistances observed were to those antimicrobials frequently employed in veterinary practices. We recommend more restrictions on the irrational use of antibiotics and public awareness activities should be undertaken to alert the public to the risks of the unnecessary use of antibiotics. Also, the study recommends that PCR should be used for rapid and sensitive detection of Salmonella.

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تواجد وتوصيف أنواع السالمونيلا المعزولة من بدارى التسمين

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قد أجريت هذه الدراسة لتحديد مدى انتشار السالمونيلا في مزارع بدارى التسمين في محافظة الدقهلية، مصر، حيث تم جمع ١٠٠٠ عينة من ٢٠٠ دجاجة من بدارى التسمين (٤٠ سليم ظاهريا - ٨٠ دجاجة مريضة – ٨٠ دجاجة حديثة النفوق). هذه العينات شملت الكبد، الأعور، دم القلب ، الطحال والكلى. من خلال شكل المستعمرة ، التعرف المجهري والبيوكيميائى للمعزلات أظهرت وجود ٣٧ من أصل ٢٠٠ الدجاج (١٨,٥٪) تمثل: ٣ من الدجاج السليم ظاهريا (٧,٥٪)، و ٢١ من الدجاج المريض (٢٦,٢٥٪) و ١٣ من الدجاج حديث النفوق (١٦,٢٥٪). أظهرت معدل استرداد السالمونيلا من الأعضاء الداخلية المختلفة بنسبة عالية من الكبد، الأعور ، الطحال ، القلب ثم الكلى (٩,٥٪)، (٥,٥٪)، (٤,٥٪)، (٣٪) و (٢٪) على التوالى. و باجراء التصنيف السيرولوجى لعترات السالمونيلا المعزولة من الدواجن تم تحديد الانواع المصلية التالية : سالمونيلا انتريتيدس (٨) ، سالمونيلا ماكسلس فيلد (١)، سالمونيلا وين جروف (٢) ، سالمونيلا اينجيدي (١)، سالمونيلا ريسين (٣)، سالمونيلا ديربى (٢)، سالمونيلا فيجلى (٢)، سالمونيلا ماغيرافيلت (١)، سالمونيلا بيرتا (٢)، سالمونيلا انتريكا تحت نوع السلامى (١)، سالمونيلا جويليتابي (١)، سالمونيلا بليجدام (١)، سالمونيلا كنتاكي(٥)، سالمونيلا نيوبورت (٢)، سالمونيلا أجونا (٢) وسالمونيلا فيرشو(٢) . وقد وجد أن الجنتاميسين والسيبروفلوكساسين وسلفات الكولستين والانروفلوكساسين أكثر المضادات الحيوية تأثيرا فى حين أن الاريثرومايسين و الفلومكوين كانا أكثر المضادات الحيوية مفاومة ضد المعزولات. كما تم اجراء اختبار تفاعل البلمرة المتسلسل لستة عترات (سالمونيلا انتريتيدس ، سالمونيلا ماكسلس فيلد ، سالمونيلا ريسين ، سالمونيلا ديربى ، سالمونيلا ماغيرافيلت ، سالمونيلا انتريكا تحت نوع السلامى) للكشف عن وجود جينات (invA، sopB ، stn) وقد تبين تواجدهم بنسبة ١٠٠%.