# Bacteriological and molecular studies on *Salmonella* in chickens

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**Abstract**

A total 396 samples from 132 broiler showed diarrhea and common clinical signs (samples from liver, heart blood and intestinal content) were collected from Port Said governorate. All samples were subjected for isolation and identification of salmonella. *Salmonella species* were recovered in 121 (30.5%) from all examined samples according to the traditional methods . 25 samples from broiler chicken which showed typical post mortem picture for salmonella infection and confirmed by biochemically traditional methods were subjected to API 20E system for qualification that rapid test. Serotyping of *Salmonella* isolates could be identified into *Salmonella enteritidis*(1,9,12;g,m;–), *Salmonella kentuckey* (8,20;I;z6), *Salmonella bloemfontein* (6,7;e,n,x,z42) and S*almonella archavalenta* (4,(15),12,a;1,7).

The result of sensitivity of the four *Salmonella* strains to 13 antimicrobial agents revealed that *Salmonella* isolates were sensitive to choloramphenicol, gentamycin, ofloxacin variable to ampicillin, enrofloxacin, naldixic acid, sulfa/trimethoprim and resistant to erythromycin and lincomycin**.**

Theses identified Serotyping of *Salmonella* were further confirmed using polymerase chain reaction technique and SYBR Green real-time PCR for detection of *Salmonella* invA gene that present only in *Salmonella* species as a rapid and more sensitive technique. SYBR Green real-time PCR was conducted to the four identified *Salmonella* serovars to detect the difference in melting temperature. It showed that *S.*enteitidis Time Product1 (-R'(T) at 82, S. *bloemfontein* at 77.63, *Salmonella kentucky at* 83.51 and *Salmonella arechavaleta at* 81.92.

**Key Word*:*** *Salmonella*; API; invA gene; SYBR Green; antimicrobial drug

## Introduction

*Salmonella* remains one of the leading causes of human foodborne disease outbreaks, and is usually associated with the consumption of poultry products ***(Alcaine et al, 2007; Scallan et al, 2011and Fernandez et al, 2012),*** This

organism cause substantial economic loss resulting from mortality, morbidity, and poor growth of infected animals, poultry and couse serious problem for the food industry ***(Banavandi et al, 2005).*** Conventional procedures used to identify *Salmonella* are time-consuming, labor intensive and requires high quality of biochemical materials ***(McQuiston et al., 2004; Gallegos-Robles et al., 2008).*** Amplification of invA gene of *Salmonella* has been reported as a suitable target for PCR amplification, with potential diagnostic applications ***(Li et al, 2013).***

API 20E and invA PCR for the identification of *Salmonella enterica* were clarified by ***Nucera et al (2006).*** Sybr Green Real -Time PCR is a useful tool when a large number of samples have to be analyzed because it is less expensive ***(De Medici et al, 2003; Elizaquivel and Aznar, 2008; Fenicia et al, 2007 and Fricker et al, 2007).***

## Material and Methods

Three hundred and ninety six samples of clinically diseased broiler showing diarrhea(132 each of liver, heart blood and intestinal content) were collected from Port Said governorate

*Media used for isolation of Salmonella species*: Enrichment medium, Rappaport vassiliadis R10 broth (Difco) and Selenite F. broth (Difco) , Selective and differential plating solid media MacConkey agar ( Biolife ), XLD (**Toply House**). and *Salmonella Shigella* agar S.S (Oxoid) ***(Cruichshank et al, 1982).*** *Media used for biochemical identification, preservation and detection of Motility*: Triple sugar iron agar. (Toply House), Simmon's citrate agar. (Oxoid), Christensen' urea agar base. (Oxoid) and Semisolid nutrient agar 0.4% ***(Cruichshank et al, 1982)***

*Media for antibiotic sensitivity test:* Muller Hinton agar (Difco). Antibiotic discs were obtained from (Oxoid),According to WHO

Reagents and Solutions for biochemical tests according to ***Cruickshank et al (1982).*** API®20E (bioMérieux® SA, 692080 Marcy r E toile, France, Lot. 1002016630)

Diagnostic *Salmonella* antisera: Polyvalent and monovalent *Salmonella* antisera (denka company of antisera) were kindly supplied from Animal Health Research institute, Doki, Giza .According to K–White scheme ***(Kauffman, 1974)*** as described by ***Edwads and Ewing (1972)*** to determine the "O" and "H" antigen .

Reagents and media used for invA gene PCR detection according to ***Sambrook et al (1989).***

Oligonucleotide primers used in cPCR. Two pairs of primers were supplied from metabion (Germany).

They have specific sequence and amplify specific products.

**DNA Molecular weight marker: Gel Pilot 100 bp ladder** (cat. no. 239035) supplied from QIAGEN. Number of bands: 6, Size range: 100-600 bp.

Detection of invA gene: according to **Sambrook et al (1989),** Cycling conditions of the primers during ***al (*1999).**

cPCR according to **(Oliveira *et al,* 2003).** Detection of S.enteritidis using SYBR Green real-time PCR: according to **Akbarmehr et al, 2010**. Cycling conditions for SYBR Green real time PCR of *S. Kentucky* according to Quantitect SYBR Green PCR kit and **Soumet *et***

| Primer | Target gene | Primer sequence (5'-3') | Length of amplified product | Reference |
|---|---|---|---|---|
| 139 | *invA* | GTGAAATTATCGCCACGTTCGGGCAA | 284 bp | **Oliveira *et al*., 2003** |
| 141 | | TCATCGCACCGTCAAAGGAACC | | |

| Agent | Primer | Target gene | Primer sequence (5'-3') | Length of amplified product | Reference |
|---|---|---|---|---|---|
| **S.Kentucky** | **fli 15** | **fliC** | CGGTGTTGCCCAGGTTGGTAAT | 559 bp | **Soumet et al., 1999** |
| | **Tym** | | ACTCTTGCTGGCGGTGCGACTT | | |
| **S.enteritidis** | **SEFA2** | **sefA** | GCAGCGGTTACTATTGCAGC | 310 bp | **Akbarmehr et al., 2010** |
| | **SEFA4** | | TGTGACAGGGACATTTAGCG | | |

## Results

121positive sample out of 396(30.5%) from clinically diseased birds showed diarrhea, clinical signs and postmortem lesions suspect salmonella infection. 25 samples obtained from heart blood collected from broiler which have typical post mortem picture and identified by traditional methods confirmed by biochemical tests were subjected to API 20E test for comparing the results with the traditional one 21out of 25 samples with percentage 84% were belong to *Salmonella* spp.

Serotyping of *Salmonella* revealed 4 isolates were serologically identified from 21 ( according to difference in biochemical testing of API) according to **(White-Kauffman-Le-Minor scheme 9th edition 2007)** using diagnostic *Salmonella* antisera polyvalent O and polyvalent H antisera by slide agglutination test

Antibiogram of isolates against different antimicrobial agents using disc diffusion technique was according to (**CDC and WHO 2003)**

Antibiogram of *Salmonella enteritidis* showed high sensitivity

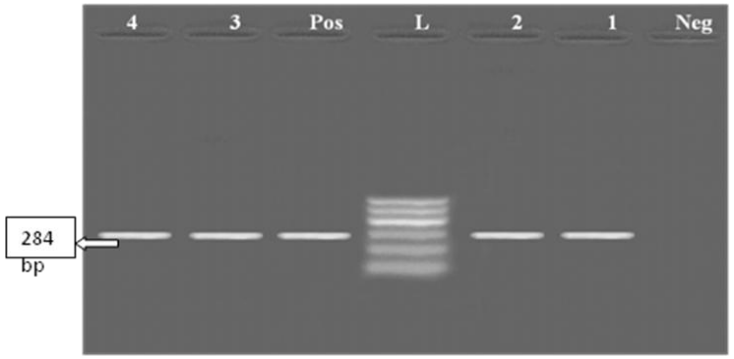
to Ampicillin, Chloramphenicol, Enrofloxacin, Gentamycin, Naldixic acid, Neomycin, Tetracyclin, Sulfa/trimethoprim, Ciprofloxacin and Ofloxacin ,while *Salmonella Kentucky were* highly sensitive for Chloramphenicol and Gentamycin only. The antibiogram of *Salmonella Bloemfontein* were highly sensitive Ampicillin, Chloramphenicol, Enrofloxacin, Gentamycin, Naldixic acid,Tetracyclin, Sulfa/trimethoprim, Ciprofloxacin, Ofloxacin and cephhadrine while *Salmonella arechavaleta* were sensitive to Ofloxacin and cephhadrine. A PCR assay targeting invA gene of *Salmonella* was standardized for rapid detection and confirmation of *Salmonella* isolates. The assay relied on specific amplification of a 284 bp product

In the SYBR Green I real-time PCR, the amplification of the DNA target is measured in terms of the increment in the quantity of fluorescence determined at the end of each amplification cycle. In brief, SYBR Green I binds to the minor groove of dsDNA, greatly enhancing the fluorescence. The results of SYBR Green real time PCR were detected the difference of the four identified *Salmonella* strains in the time product.

The result of SYBR Green of *Salmonella enteritidis* and *Salmonella Bloemfontein, kentucky* and *Salmonella arechavaleta*

**Table ( 1 ):** *Serological identification of the four suspected Salmonella isolates*

| Serotype | Somatic antigen (O) | Flagella (H) antigens | |
|---|---|---|---|
| | | Phase 1 | Phase 2 |
| *S. enteritidis* | 1,9,12 | g, m | – |
| *S. kentuckey* | 8,20 | I | z6 |
| *S. bloemfontein* | 6,7 | b, e, n, x | z42 |
| *S. archavalenta* | 4, (5),12 | a | 1,7 |




**Photo(1):** Electrophoresis of invA gene in the four isolates by PCR products on 1.5% agarose

**Table (2):**The time product after using SYBR Green dye on *Salmonella enteritidis* , *Salmonella Bloemfontein, Salmonella kentucky* and *Salmonella arechavaleta*

| Well name | Dye | Tm Product1 (-R'(T)) |
|---|---|---|
| Negative(S. *bloemfontein*) | **SYBR** | 77.63 |
| *S.enteitidis* | **SYBR** | 82.00 |
| Pos. *S. enteitidis* | **SYBR** | 82.53 |
| Negative(S. *arechavaleta*) | **SYBR** | 81.92 |
| S. *kentucky* | **SYBR** | 83.51 |
| Pos. *S. Kentucky* | **SYBR** | 83.51 |

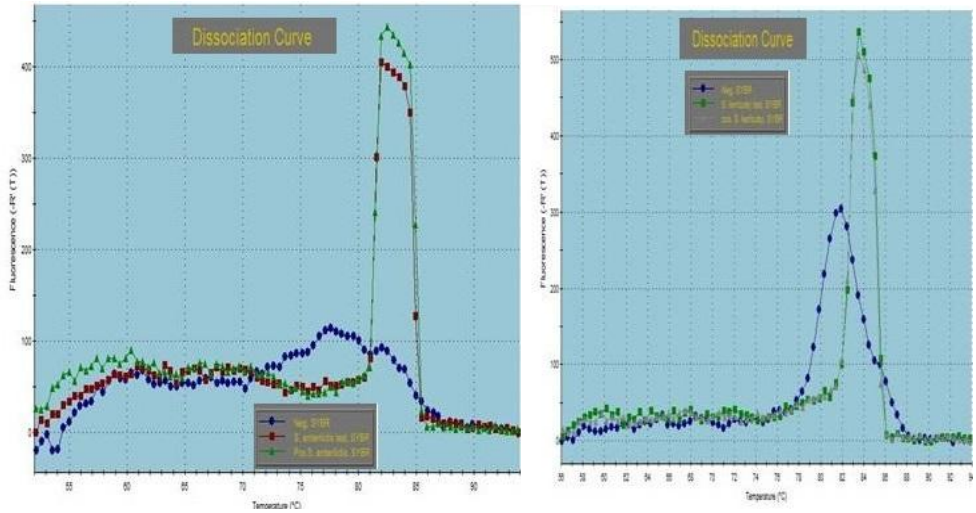



**Photo (2):**Time product using SYBR Green dye on *Salmonella enteritidis* , *Salmonella Bloemfontein, Salmonella kentucky* and *Salmonella arechavaleta*

**Discussion**

*Salmonella* causes a serious health problem in developing countries through a wide range of human diseases such as enteric fever, gastroenteritis and bacteremia ***(Banavandi et al, 2005)***. The majority of human *Salmonellosis* cases are caused by the consumption of contaminated poultry products ***(Geimba et al, 2004)***. All samples collected from suspected birds showed symptoms were similar to those described by ***(Gast, 2003)***. Gross lesions were similar to that reported by ***(Gast, 2003)***. For the isolation of *Salmonellae*, enrichment was necessary which done using Rappaport- vassiliadis, same results were detected by ***(Iwade et al, 2006)***. All samples were firstly inoculated into Rappaport - vassiliadis t broth, (which enriched small number of *Salmonella* in contaminated samples) and

incubated for 24 hours, before plating on solid media; as these enrichments and plating media have been successfully used by ***Harvey and Price (1975)*** and ***Nassib et al (2003)***. Similar colonial appearance was reported by and ***Cruickshank et al (1982)*** and ***Gast et al (2004)***.

Incidence of *Salmonella* isolation from organ samples were 30.5% from heart blood , liver and caecal content that collected from 132 broiler chickens. Suspected isolates were confirmed biochemically according to ***(Cruickshank et al,1982)***.The obtained results were in agreement with ***(Bailey et al, 2005 )*** who reported that *Salmonella* positive samples ranged from 8% to 34% due to wide spread of *Salmonella*. This higher incidence suggesting that *Salmonella* spp. indicating wide spread and potential hazard of interspecies sharing of this organism and require more epidemiological survey.

*Salmonella* was localized with highest incidence in intestine and caecal content then followed by liver and heart as *Salmonella* passed from digestive tract firstly then followed by parenchymatous organs these results were in agreement with data reported by ***Berthelot-Herault et al (2003)***.

The present study showed that isolation *Salmonella* according to the reading of API 20E system were 84% (21 out of 25). These percentages were closer to ***Saeed et al (2011)*** but in disagreement with ***Nucera et al (2006),*** this may be attributed to the difference in sampling procedure. Serological tests should be conducted to confirm biochemical testing. Serological and molecular test can be applied on the pure culture to provide a definitive confirmation of the isolated strains. *Salmonella*e possess antigens designated somatic (O) and flagella (H) which may be identified by specific typing sera, and the serovar may be determined by reference to the antigenic formulae in the Kauffman–White scheme .Our results of serological identification agreed with ***Brenner et al (2000).*** The isolates confirmed and serotyped as S.*enteritidis, S.kentucky, S.bloemfontein* and S.*arechavaleta*. As illustrated in table(1 ),the antigenic formula of *Salmonella enteritidis* was(O1, 9, 12; g, m; –), *Salmonella kentuckey*(O8,20;I,z6), *Salmonella bloemfontein*(O6, 7; b; e, n, x; z42) and *Salmonella arechavaleta*(O4, (5), 12; a; 1, 7) . These results were agreed with ***Grimont et al (2007)*** *S. bloemfontein and S. arechavaleta* were isolated for the first time in Egypt.

Concerning sensitivity to antibiotics the isolated 4 strains were examined against 13 antibiotic discs. The MICs (Minimum Inhibitory Concentrations) data demonstrated very high level of resistance to erythromycin and

lincomycin. Previous studies on *S. enteritidis*, isolated from poultry had also demonstrated 100% resistance to erythromycin and lincomycin. The development and spread of antimicrobial resistance to erythromycin and lincomycin may be linked to selection pressure caused by excessive use of this drug. *Salmonella* resistance at varying concentrations of erythromycin has also been reported by ***Sultana et al (1995).*** The higher resistance rates of *S. enteritidis* to erythromycin and lincomycin suggest limiting use of therapeutic potentials of these antimicrobial agents. From the antibiogram for the four strains our study showed that were resistant to erythromycin and lincomycin antimicrobial agents this were in agreement with ***Randa et al (2006).***

Antimicrobial resistance in *Salmonella* strains is generally encoded by plasmid, which has been acquired as consequence of antibiotic pressure in humans and veterinary medicine; however, due to the fluidity of resistant plasmids and transposes, antimicrobial drug resistance pattern cannot be recorded as satisfactory method for discriminations within serovars ***(Olsen etal., 1992).*** An inevitable side effect of antibiotic use, which was associated to the adaptability of bacteria and microbial genome evolution, was the emergence and dissemination of resistant bacteria. Resistant commensally bacteria of food animals might contaminate, like S. enteritidis (zoonotic bacteria), poultry products, thus reaching the intestinal tract of humans along with contaminated food. Antimicrobial resistance can involve not just obvious pathogens but also commensal bacteria, which may act as an enormous reservoir of antimicrobial resistance genes**.** Thus, antimicrobial use in medicine and agriculture affects the general ecology of bacterial communities, including interactions between bacteria and their environment but also mechanisms by which antimicrobial resistance genes spread and persist. Their use, especially in food animals, can also have adverse effects on human health ***(O,Brien, 2002)***. Resistance can be caused by a large number of mechanisms, involving decreased antibiotic accumulation, physical modification or destruction of the antibiotics, and alteration of the enzyme target of antibiotic action. Recently, a mechanism of resistance involving the active efflux of antibiotics by multidrug efflux pumps was also elucidated ***(Soto et al, 2003)***.

In contrast to traditional methods, the use of PCR is one of the most sensitive, specific and rapid tests through rapid detection for invA gene which is specific for genus *Salmonella*. PCR-based techniques can be applied to detect culturable and non-culturable *Salmonella* in samples within hours Instead of days required in conventional biochemical assays ***(Abd El-***

***Haleem et al, 2003)***. So, at the next step, PCR technique was used to confirm the previous results. In this work molecular genetics study has been carried out to identify the genetic character of *Salmonella* by using invA gene specific PCR ***(White et al, 2002)*** .

A PCR assay targeting invA gene of *Salmonella* was standardized for rapid detection and confirmation of *Salmonella* isolates. The assay relied on specific amplification of a 284 bp product as shown in photo (1). All the biochemically confirmed isolates of *Salmonella* were tested for amplification of invA gene by PCR, the results agreed with ***Kaushik et al (2014)*** who reported that *Salmonella* positive by predicted a 284 bp DNA fragment when all strains were subjected to *Salmonella*- specific gene (inv A).

The SYBR® Green chemistry was preferred to the commonly used TaqMan® chemistry since SYBR® Green assays present the advantage of allowing the use of only two primers in the conserved region surrounding the more polymorphic sequence. In addition, SYBR® Green assays are cheaper and permit post-amplification verification through specificity by melting temperature analysis ***(Postollec et al, 2011).***

The SYBR® Green qPCR assays for *Salmonella* detection developed and validated in this paper could be combined with the four assays for *Salmonella* detection previously described by ***Barbau-Piednoir et al ( 2013)*** and run all together on a single plate. Since this approach is modular, detection assays can be added to detect a wider range of foodborne pathogens or emerging pathogens. The only requirement is to develop an assay able to be run with the same conditions, allowing its use in high throughput on the same 96-well plate.

Table (2) &photo (2) clarified that isolated *S.enteitidis* test was confirmed by SYBR Green real time PCR showed that Tm Product1 (-R'(T) at temperature 82 compared to control positive (*Salmonella enteitidis*) at 82.51 and negative with *S. Bloemfontein* at 77.63. *S.kentuckey* was confirmed by SYBR Green real time PCR Tm Product1 (-R'(T) at temperature at 83.51 compared to control positive *Salmonella kentucky at* 83.51 and negative *Salmonella arechavaleta at* 81.92.

From this study we conclude that *Salmonella* are the main cause of economic losses resulting from mortality, morbidity, and poor growth of infected birds, Also, considered one of human food borne disease outbreaks. so, the detection and eradication of *Salmonella* from poultry farms is of primary importance. Also the traditional methods for identification of *Salmonella* were more accurate than API 20E system, but were time-consuming,

labor intensive and require high quality of biochemical materials. Detection of *Salmonella* invA gene that present only in *Salmonella* species is one of the most sensitive, specific and rapid tests. Such data is essential for developing appropriate treatment of salmonellosis. Periodical microbiological examination against *Salmonella* species in broilers must be done. The need for continued surveillance emphasized to determine local antimicrobial susceptibility data to identify changing pattern of resistance to avoid miss-use of antibiotic and resistant strains against the antimicrobial agents.

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