Flagellar Biofilm in relation to Flagellar Gene for Virulence of Salmonellae in Chicken

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Abstract

The present study was conducted to determine the effect of Salmonella infection on 152 chicken samples of different ages in two seasons cold (winter and spring) and hot (summer and autumn) seasons at Dakahlia Governorate. Chicken were examined clinically and postmortem and the samples were taken from livers, caecum, spleen and small intestine for Salmonella isolation. *S*.Typhimurium were examined for its ability to form biofilms on the wall of glass tubes and also Polymerase chain reaction (PCR) was used for detection of flagellar gene (flAg) of *S*.Typhimurium which is an essential gene required for motility of Salmonella. Salmonellae were isolated from (27) out of (152) chickens that were examined (17.8%) with high incidence during hot seasons (25%) in contrast to cold seasons (10.5%), the rate of recovery of Salmonellae from the different internal organs showed that the recovery rate was; liver (12.5%), caecum (8.55%), spleen (7.24%) and small intestine (3.29%). Serotyping of isolates revealed that *S. Typhimurium* represented 13.8% (21 isolates) and *S. Kentucky* 3.95% (6 isolates) from the examined samples, *S.Typhimurium* and *S*.Kentucky were isolated from liver while *S.Typhimurium* is the only serovar which isolated from small intestine, caecum and spleen. PCR-polyacrylamide gel electrophoresis detected flagellar gene of *S. Typhimurium* with amplicon 375 bp and *S.Typhimurium* had the ability to form biofilms on glass surface of the test tubes but, *S. Pullorum* did not have the flagellar gene as non motile serotype (reference strain from Namiro Center in Egypt) so was not detected by PCR and this proved the relation between motility and biofilm formation. Results of antibiotic sensitivity test of *S.Typhimurium* and *S.Pullorum* proved that there is no correlation between biofilm formation by flagellae and antibiotic susceptibility.

Introduction

The consumption of poultry meat continues to rise in both developed and developing countries throughout the world. In 1990, global production of broiler chicken reached 40 billion for the first time and by 2020, poultry is predicated to become the overall meat of choice *(Biligli, 2002)*. Poultry and poultry products had been incriminated in the majority of traceable food-borne illnesses caused by bacte-

ria, although all domestic livestock are reservoirs of infection (*Thorns, 2000*). Among the food-borne pathogens the genus *Salmonella* is one of the most common causes of foodborne infections worldwide (*Baird-Parker, 1990*). More than 2,500 different serovars of *Salmonella enterica* had been identified and most of them had been described as the cause of human infections, but only a limited number of serovars are of public health importance. Most reports have mentioned *Salmonella enterica* serovar Typhimurium and *Salmonella enterica* serovar Enteritidis as the most common causes of human salmonellosis world wide (*Scuderi et al., 2000*). An infection with Salmonella usually starts by ingestion, followed by colonization in the intestine. After colonization, Salmonella is able to penetrate the mucosal epithelium which results in a systemic infection, with colonization of the spleen and liver (*Henderson et al., 1999*). Biofilms are aggregations of microorganisms adherent to each other and/or to a surface and embedded in a matrix of exopolymers (*Costerton et al., 1999*). The occurrence of biofilms in food processing environments can cause post-processing contamination leading to lowered shelf life of products and transmission of the diseases (*Mahdavi et al., 2008*). The food-borne pathogen *S. enterica*, as well as other members from family *Enterobacteriaceae*, form biofilms on biotic and abiotic surfaces during their natural life cycles (*Prouty and Gunn, 2003 & Ledeboer and Jones, 2005*). *S.* Typhimurium and closely related bacteria, structural genes required for flagellar motility and pili, and the production of colanic acid, lipopolysaccharide, enterobacterial common antigen lipid II and cellulose, all contribute to the formation of biofilms on different biotic and/or abiotic surfaces (*Boddicker et al., 2003; Solano et al., 2002 and Zogaj et al., 2001*).

Oliveria et al., (2003) and Fratamico, (2003) revealed that PCR method was high specific and sensitive and more importantly a less time-consuming procedure than standard microbiological techniques for detection and identification of Salmonella. Moreover PCR method was more accurate and used as a confirmation method for somatic serogrouping with polyvalent antisera as serogrouping was not possible when Salmonella isolates lack O-antigen (rough strain) or lack both O and H antigens (*Roy et al., 2002*). Detection and monitoring of drug-resistant salmonellae are important to substantiat the choice of antibiotics for the treatment of clinical Salmonellosis and to assess the risk of exposure of multiple drug resistant strain (*Yang et al., 2002*). The present study was directed to study: The incidence of Salmonella in chicken, detection of flagellar gene of Salmonella using Polymerase Chain Reaction, the ability of *S.* Typhimurium to form biofilm and its relation to flagellar motility in comparison to *Salmonella* Pullorum and the relation of sensitivity to antimicrobial agents with biofilm formation by flagellar gene.

Materials And Methods

A total of 76 freshly dead and 76 diseased living birds of different ages

(1day, 33, 67 and 150 days) were collected from different farms in both summer and winter seasons located at *Dakahlia* Governorate and subjected to clinical and postmortem (P.M) examination as well as for isolation and identification of Salmonellae from tissue samples including liver, caecum, spleen and small intestine. The birds showed signs of septicemia, retarded growth, depression, profuse watery diarrhea and accumulation of fecal matter around the vent. The freshly dead birds showed bronze discoloration and enlargement of liver, spleenomegaly, inflammation of intestine and caecum and unabsorbed yolk sacs in young chicks. All tissue samples were collected and handled aseptically to prevent cross contamination using sterile sampling materials (bags, knifes, flasks, scissors and forceps).

Detection of Salmonella by conventional method was done according to *Iso 6579 (2002)* with some modifications.

Selective enrichment of Salmonella in broth: as Selenite F broth or Rappaport-Vassiliadis Soya broth (RVS) or Muller-kauffmann tetrathionate novobiocin broth (MKTTn broth).

Colonization of Salmonella on selective differential solid media: *Salmonella Shigella* agar (S-S agar), Xylose Lysine Deoxycholate agar (XLD), Brilliant Green agar (BGA agar), Hekton Enteric agar and MacConkey's agar.

Identification of suspected Salmonella colonies: Culture characters:
On *Salmonella Shigella* (S-S) agar Salmonella produce colorless colonies with black centers due to H_2S production, on Xylose Lysine Deoxycholate agar (XLD): Salmonella produce yellow colonies at first which later on changes into red with black center due to H_2S production, on Brilliant Green agar (BGA agar): Salmonella produce pink colonies, on Hekton Enteric agar: Salmonella appeared blue-green colonies with or without black centers and on MacConkey's agar: Salmonella produce colourless colonies because they are non lactose fermenters exept *S.*Arizonae.

Microscopic examination: Gram's stain was prepared and used as described by *Cruickshank et al. (1975)* to study the morphology.

Detection of bacterial motility was done by using semisolid nutrient agar medium (0.4%) (soft agar): according to *(Cruickshank et al., 1975)* and modified semisolid Roppaports Vassiliadis medium (MSRV) according to *De Smedt and Bolderdijk (1987).*

Biochemical Identification was done according to *Quinn et al. (2002)* on TSI (Triple sugar iron agar), urease, Lysine iron, Indole reaction, Methyl red test and Voges-proskauer.

Serological identification: The obtained biotyped isolates were serotyped in Animal Health Research Institute, Dokki, Giza and Ministry of Health, Centeral Health Laboratories, Abdien, Cairo using: Polyvalent "O" *Salmonella* antisera, monovalent "O" *Salmonella* antisera and "H" *Salmonella* antisera.

Detection of *Salmonella* Typhimurium FlgA gene using Polymerase chain reaction (PCR): *(Simonelli et al., 2009 and Malorny et al., 2004).*

Materials used for nucleic acid extraction by QIAamp®DNA Mini Kit (Cat. No. 51304 Qiagen), PCR Master Mix for conventional PCR: Through using of PCR 1.1x ReddyMix TM Master Mix (Thermo Scientific) with Cat. No. (AB0575/LD-A) that is ready to use, Enzyme Source: Thermus aquaticus and Kit Components: Each vial contains 5 ml of a 1.1x working concentration PCR Master mix containing:1.25 units (Thermoprime Plus DNA Polymerase), 75 mM Tris-HCL(PH 8.8 at 25°c), 20 mM $(NH_4)_2SO_4$, 1.5 mM MgCl2, 0.01% (V/V) Tween® 20 , 0.2mM each of dATP, dCTP, dGTP and dTTP Precipitant and red dye for electrophoresis. It is stored at -20°C until ready for use for up to 1 year. Repeated freezing and thawing should be avoided. The vial can be stored at 4°c for up to 1 month.

Oligoneucleotide primers were designated according to Integrated DNA Technology purchased from Georg August Universitat-Berlin- Germany and used for amplification of the flagellar gene (flgA) of *S. Typhimurium*. The primers were received in lyophilized form and resuspended in Tris/EDTA (TE) buffer to reach a final concentration of 100 pmol/µl. These primers were suspected to amplify specific segment of 375 bp. A detailed descriptions of the designed oligonucleotide primers is Forward (5`TTCCCATCAGAATCGACGGTT CCA 3`) and Reverse (5`GAATGTGCAAGCGACCGGCA ATTA 3`).

Material used for agarose gel electrophoresis: Agarose 1.5% according to *(Sambrook et al., 1989)*, Ethedium bromide solution 10 mg / ml according to *(Sambrook et al., 1989)*, Tris borate EDTA (TBE) electrophoresis buffer(1x) according to *(WHO, 2002)*, Gel loading buffer (6 x stock) according to *(Sambrook et al., 1989)* and DNA Molecular weight marker: Gel Pilot 100 bp ladder (Cat. No. 239035) supplied from QIAGEN with size range 100-600 bp.

DNA extraction and purification: By QIAamp®DNA Mini Kit (Cat. No. 51304-Qiagen) used according to manufacturer's instructions.

Amplification and cycling protocol for conventional PCR : Using of PCR 1.1x ReddyMix TM Master Mix (Thermo SCIENTIFIC) with Cat. No. (AB0575/LD-A) for conventional PCR (uniplex PCR).

Detection of PCR products: *(Augustynowicz et al., 2000)* Aliquots of amplified PCR products were mixed with gel loading buffer and electrophoresed in 1.5% agarose gel.

Biofilm formation:*(Hee Kim and Wei, 2009; Head and Yu. 2004).*

A loopful from each of isolated *S. Typhimurium* and *S. Pullorum* (reference strain from Namiro Center in Egypt) was inoculated separately in 5 ml Rappaport-Vassiliadis Soya broth (RVS) at a ratio 1:10 (sample : broth) and incubated at 41 °C for 24 hours. Then 1ml from the inoculated broth was transferred into another sterilized 4 ml Rappaport-Vassiliadis Soya broth (4 tubes), and one tube from broth was used as a control (not inoculated) and then incubated at 41 °C for 5 days.

The inoculated broth was discarded carefully, stained with crystal violet

1% stain for 15 minute and then the stain was discarded. The tubes were photographed to determine the ability of *S. Typhimurium* and *S. Pullorum* isolates to make biofilms on their wall.

Sensitivity of Salmonellae to antimicrobial agents: *S. Typhimurium* and *S. Pullorum* were tested for their antimicrobial sensitivity to various antibiograms by the agar disc diffusion method according to *Bauer et al. (1996).*

Results

Table (1): Incidence of Salmonella infection in chickens

Number of examined chicken	Number of positive	Percentage of positive %
152	27	17.8

Table (2): Seasonal variation of Salmonella infection in chicken

Season	Number of examined chicken	Number of positive	Percentage of positive %
Hot season (summer and autumn)	76	19	25
Cold season (winterand spring)	76	8	10.5
Total	152	27	17.8

Table (3): Rate of recovery of Salmonella from internal organs

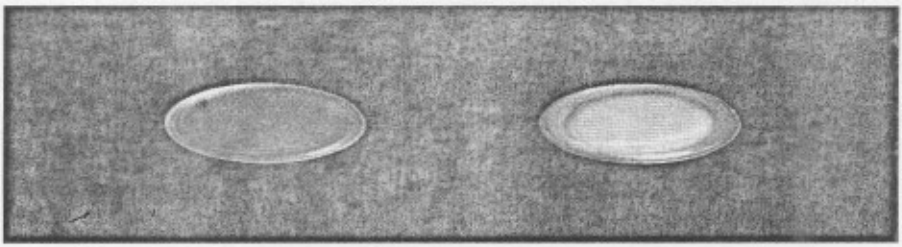
Examined organs in 152 chicken	Number of positive	Percentage of positive %
Liver	19	12.5
Caecum	13	8.55
Spleen	11	7.24
Small intestine	5	3.29
Total	48	7.89

Table (4): Salmonella serovars recovered from bacteriologically examined chicken

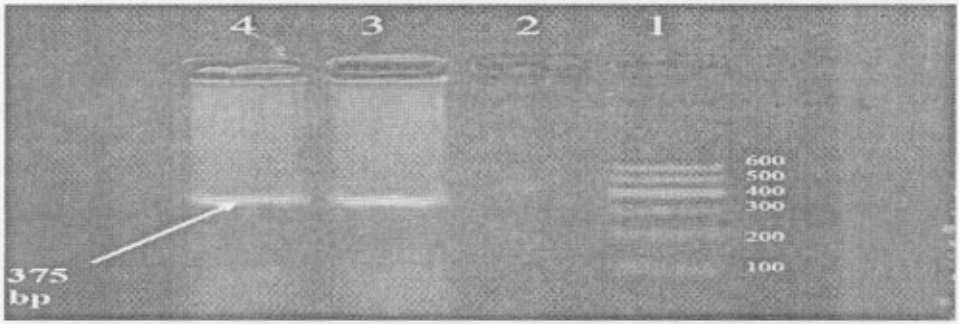
The infected serovar	Number of positive chicken	Percentage of positive%
*S.*Typhimurium	21/27	77.8
*S.*Kentucky	6/27	22.2

Table (5): Salmonellae serovars isolated from examined chickens

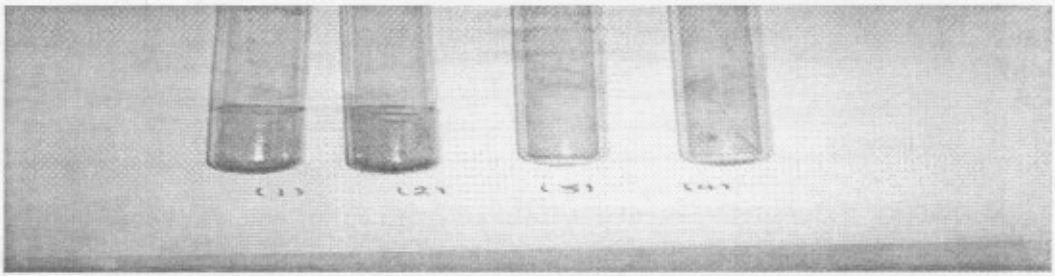
Examined organs	Number of examined chicken	Strain	Number	Percentage %
Liver	152	*S.*Typhimurium	15	12.5
		*S.*Kentucky	4	
Caecum	152	*S.*Typhimurium	13	8.55
Spleen	152	*S.*Typhimurium	11	7.24
Small intestine	152	*S.*Typhimurium	5	3.29



Photograph (1): Motility of *Salmonella* Typhimurium on modified semisolid Roppaports Vassiliadis medium (MSRV):
(1) : Negative plate.
(2) : Positive plate shown wide zone of growth.



Photograph (2): Amplification of the Flagellar gene (FlgA) of *S. Typhimurium*: Amplification of 375 bp was observed in the extracted DNA of *S. Typhimurium* (as shown in lanes 3 which is the isolated *S. Typhimurium* and 4 which is the positive control). No amplification was observed in *S. Pullorum* genomic DNA (as in lane 2) which was used as a negative control while lane 1 used as 100 bp molecular weight marker.



Photograph (3): Biofilm formation by *S. Typhimurium*: Tube number (1) shows biofilm formation by *S. Typhimurium* and tube number (2) is the positive control. After crystal violet 1% staining and washing with water, the attached cells of the biofilm remains in the tube and retained the stain color indicating biofilm formation. However, in the tube number (3) which has *S. Pullorum* there was no color observed after staining with crystal violet 1% and washing with water indicating lack of biofilm formation. Tube number (4) is the negative control.

Sensitivity of *Salmonella* Typhimurium and *Salmonella* Pullorum to antimicrobial agents

Antibiotic discs	*Salmonella* Typhimurium	*Salmonella* Pullorum
Chloramphenicol	S	S
Gentamycin	S	S
Ciprofloxacin	S	S
Flumequine	R	R
Lincomycin	R	R
Nalidixic Acid	R	R
Oxolinic Acid	R	R
Erythromycin	S	R
Ampicillin	R	S
Neomycin	R	S
Colistin Sulphate	R	S

R: Resistant. S: Sensitive.

The two strains were sensitive to Chloramphenicol, Gentamycin and Ciprofloxacin and were resistant to Flumequine, Lincomycin, Nalidixic Acid and Oxolinic Acid.

Discussion

Poultry products are known to be a significant reservoir for Salmonella and most important source in human infection *(Maripandi and Al-Salamah, 2010)*. In the present study, the incidence of Salmonella in chickens was 17.8% and this result may be due to contamination of the eggs in the hatcheries, vertical transmission through infected eggs from the carrier cases, contaminated feed and water or changes in the climate weather. In contrast, *Olesiuk et al. (1969)* reported that hatcheries represent a major site of horizontal infection where eggs from different laying flocks are sent for hatching. Salmonella were usually obtained from only a small percentage (0.01 to 0.05%) of fertile hatching eggs entering the incubator.

Season of the year is considered to be an important factor affecting incidence of Salmonella infection in chicken as the results revealed that there was a high incidence of Salmonella in hot seasons (25%) than cold one (10.5%). These results may be due to the stress of high ambient temperature and relative humidity which lead to deprivation of food for long periods and then decrease in the cellular immune response of the chicken. These results were almost similar to that obtained by *Martinez et al. (2004)* who reported that Salmonella contamination could vary on seasonal or temporal factors such as temperature, wind, hours of sunlight, rainfall and humidity marine environments. The present study revealed that, the most Salmonella isolates obtained were from livers of the examined chickens followed by caecum, spleen and small intestine 12.5%, 8.55%, 7.24% and 3.29%, respectively. The explanation of these results may be due to the ability of Salmonella organisms to attach and invade the ileum, then spread extra intestinally in low numbers early during the course of infection and require

sometimes to multiply and increase till reach the detectable number in the liver and other organs *(Holt et al., 1995)*. On the other hand, *Zhang et al. (2003)* explained that within animal hosts, *S.Typhimurium* actively invades the intestinal epithelial layer preferentially at the Peyer's patches. Bacteria that penetrate the epithelium are engulfed by macrophages depending on the Salmonella serovar and the animal species, the bacterium can survive and replicate within macrophages and disseminate to the mesenteric lymph nodes, spleen and liver.

The most predominant serotype of Salmonella in the present study was *S. Typhimurium* 77.2% (21 out of 27 isolates) and this is due to infection, *Salmonella enterica* serovar Typhimurium must adapt to changes in [O_2] encountered in the gastrointestinal tracts of the host *(He et al., 1999)*. However, *Guignard et al. (1992)* showed that, the most predominant isolated strains were *S.Typhimurium* (73 isolates) and *S.Entertidis* (17 isolates). *Although S. Kentucky* was the most prevalent serotype (53%) isolated by *Melendezet al. (2010)* from two pastured poultry farms and retail carcasses from a local natural foods store and a local processing plant. In this study 22.2% (6 out of 27 isolates) were recorded for *S.Kentucky* only and *Bada-Alambedji et al. (2006)* recorded that the most prevalent serovar was *S.Kentucky* 30%. In the present study Polymerase Chain Reaction method was used for detection of flagellar gene (flgA) of *S. Typhimurium* which is responsible for its motility and biofilm formation by amplification of 375 bp fragments of DNA. It was found that *S. Typhimurium* had the ability to form biofilm on the inner walls of the glass tubes after incubation in Rappaport-Vassiliadis broth (R.V) media. This result may be due to flagellar motility of *S. Typhimurium* by flagellar gene (flAg) but *S. Pullorum* was non motile & did not produce biofilm.

The flagelluar role in biofilm formation has two potential roles, the first one as a propeller used to move the cells through a liquid environment and the second one as an adhesive appendage *(Landry et al., 2006 and Lillehoj et al., 2002)*. On the other hand *(O'Toole et al., 2000)* reviewed that, biofilm formation is impaired by mutations in genes involved in flagellar mediated motility, twitching motility, synthesis of exopolysaccharides, quorum sensing, outer membrane adhesins as well as global regulators of gene expression.

In the present study, the antibiogram was carried out against 2 different Salmonella serovars one motile (*S.Typhimurium*) and the other non-motile (*S.Pullorum*) using 11 different sensitivity discs. The obtained results showed that there is no correlation between biofilm formation by flagellae and antibiotic resistance where as both of these 2 studied serotypes *S. Typhimurium* and *S. Pullorum* produced mostly similar results of antibiotic resistance and antibiotic sensitivity as the two strains were sensitive to Chloramphenicol, Gentamycin and Ciprofloxacin and resistant to Flumequine, Lincomycin, Nalidixic Acid and Oxolinic Acid. These results may be due to the comprehensive use of

antibiotics included in feeds as growth promoters. Salmonellae were found to be sensitive to chloramphenicol *(Shahata et al., 1990)*, their highest antibiotic sensitivity to ciprofloxacin (100.00% sensitivity) which was in correlation to the reports of *(Zahrei et al., 2005 and Selvaraj et al., 2010)* and also sensitive to gentamycin in similarity with *Cortinez et al. (1995)*. On the other hand, all were resistant to nalidixic acid as reported by *Fashae et al. (2010)*. However, *Shahata et al. (1990)* recorded that Salmonella isolates were sensitive to fluemquine. Also *Adams and Nelson (1968)* showed that, all the isolated strains of Salmonella were resistant to lincomycin (100%).

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الملخص العربى

البيوفيلم الحركى و علاقته بالجين الحركى لضراوة السالمونيلا فى الدجاج

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*: *المعمل القومى للرقابة البيطرية على الانتاج الداجنى بجمصة – معهد بحوث صحة الحيوان – وزارة الزراعة.*

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يعتبر ميكروب السالمونيلا من أهم الميكروبات التى تصيب الدواجن على مستوى العالم ومن ثم يعتبر من أهم مسببات الأمراض للانسان. حيث يوجد أكثر من ٢٨٠٠ نوع ممكن أن تصيب الانسان من خلال تناول الأطعمة وخصوصا الدواجن. وتتم الإصابة غالبا عن طريق تناول الأطعمة الملوثة بالميكروب .

فى هذا البحث تمت الدراسة على ١٥٢ دجاجة تم جمعها من مزارع مختلفة فى مختلف الأعمار وفى فصول السنة المختلفة وقد وجد الآتى:

تم عزل ٢٧ حالة اصابة من ميكروب السالمونيلا (١٧,٨ %) وتم التأكد منها عن طريق الاختبارات البيوكيميائية ثم التصنيف السيرولوجى للمعزولات. وبعد ذلك تم عمل اختبار البلمرة المتسلسل للكشف عن جين الحركة فى السالمونيلا. كما تم عمل اختبار لمعرفة قدرة السالمونيلا تيفميوريم على عمل فيلم بيوكيميائى فى بعض الظروف المختلفة التى يتعرض لها الميكروب كما تم عمل اختبار الحساسية لبعض المضادات الحيوية وقد أثبتت النتائج ما يلى:

وجود حزمة عند ٣٧٥ عند الكشف على جين الحركة فى السالمونيلا تيفميوريم وعدم وجوده فى السالمونيلا بلورم.

قدرة السالمونيلا تيفميوريم على عمل فيلم بيوكيميائى وعدم قدرة السالمونيلا بلورم على تكوينه مما يدل على ارتباط تكوين الفيلم مع حركة الميكروب.

السالمونيلا تيفميوريم و السالمونيلا بلورم مقاومة لعدة مضادات حيوية وهى فلوميكين و لينكومايسين و نااليدكسيك أسيد و اكسولينيك أسيد كما أنها حساسة لسيبروفلوكساسين و جينتاميسين وكلورامفنيكول.

وقد وجد أنه لا يوجد علاقة بين تكون الفيلم البيوكيميائى و الحساسية للمضادات الحيوية.