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Research Article

Antibiotic Resistance Pattern and Biofilm Genes of Different *Salmonella* serotypes Isolated from Chicken Samples

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ABSTRACT

A total of 9 *Salmonella* spp strains from avian sources were completely identified as following: *S. Entertidis, S. Hadar, S. Kentucky, S. Typhimurium and S. Heidelberg*. The current study showed that biofilm genes (*adrA, gcpA* and *csgD* genes) were detected with an incidence (8/9) 88.8%, (9/9)100% and (9/9) 100% respectively. High level of antibiotic resistance was detected in all *Salmonella* isolates evaluated. All 9 isolates were resistant to ampicillin, erythromycin and cephalothin with percentage (100%), followed by tetracycline (44.4 %), gentamycin (22.2%), ciprofloxacin and sulfamethoxazole -trimethoprim (11.1%) respectively as mentioned in (Table 3). In addition to that, the isolates showed intermediate-resistant to ciprofloxacin (55.5%) followed by gentamycin (22.2%) while All *Salmonella* isolates were susceptible to chloramphenicol (100%) and the percentage of susceptibility of the *salmonella* isolates began to decrease to be detected in sulfamethoxazole –trimethoprim(88.8%), gentamycin (55.5%), tetracycline (55.5%) and ciprofloxacin (33.3%) respectively. The current study found a strong relation between biofilm formation and antibiotic resistance.

Key words: *Salmonella*, Antibiotic, Resistant, Biofilm

INTRODUCTION

Salmonellosis is a critical animal disease transmits by food and causes a widespread outbreaks of diseases and gastrointestinal infections throughout the human world as well as rising medical and economic costs (Lee *et al*., 2015). Infected food products, poultry, pigs, contaminated drinking water, ruminants and direct contact with infected animals are the major causes of *Salmonella* infections (European Food Safety Authority and European Centre for Disease Prevention and Control, 2019, Mezal *et al.*, 2014). *Salmonella* is the most basic reason of high mortality and morbidity rates in poultry farms, it has the ability to persist in the dry faeces, feed and environment for several years. The persistence of *Salmonella* is attributed to its capacity to form biofilm (Bordoloi *et al*., 2017). *Salmonella* has exhibited the capacity to create biofilms on abiotic surfaces such as plastic, rubber, cement, glass, and stainless steel (Moretro *et al*., 2009).The formation of biofilm on equipment and tools is thought to be the origin of pathogenic bacteria that rises the risk of food product contamination in food processing systems (Shi and Zhu,2009).A biofilm formed a mucoid substance known as an extracellular matrix (ECM), comprised of polysaccharides, proteins, and nucleic acids, the security of the bacterial community against the dangers of external (e.g., antibiotics) and internal (e.g., innate immune system) factors is the primary function of matrix in human infections as bacterial cells inside a biofilm are encapsulated (Parsek and Fuqua, 2004). While these general functions are related with most microbial biofilms, the individual ECM components often possess unique properties for the bacterial community and with regard to the host (Gunn *et al*., 2016). Biofilm formation by *Salmonella* spp is controlled by a highly complex regulatory network, which includes different genes. The csgD gene is part of the csgDEFG operon, the main control unit in biofilm formation by *Salmonella*, which positively regulates csgBA and adrA expression (Steenackers *et al*., 2012).The spread of antibiotics resistance between different microorganisms is representing a major intimidation to public health, various serotypes of *Salmonella* show a relatively high antimicrobial resistant (Mayrhofer *et al*.,2004) One of the serotypes have showed greatest antimicrobial resistance is *S. Typhimurium* (Alvarez-Fernandez *et al*., 2012). The present study aimed to detect certain biofilm-producing genes (*adrA,gcpA* and *csgD*) in *Salmonella* spp isolated

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from avian sources as rapid and accurate confirmation tool rather than conventional methods. In addition to, assess the antibiotic resistance and determine the link between biofilm formation and antimicrobial resistance.

MATERIALS AND METHODS

Bacterial strains (Source, isolation and identification)

Nine *Salmonella spp* strains from avian sources were randomly selected from Microbiology laboratory in National Research Center and were cultured on a nonselective pre-enrichment, followed by selective enrichment and plating onto selective and differential agars. Suspected colonies were confirmed biochemically by using API 20E test kit (bioMérieux, Inc., France), the plastic strips holding twenty mini-test tubes were inoculated with the saline suspensions of the cultures according to manufacturer's directions according to (Elgohary *et al*., 2017).

Serological identification

It was carried out using White Kauffmann-Le Minor scheme as described by (Elgohary *et al*., 2017). The typing antisera were obtained from Denka Seiken Co. Ltd, Tokyo, Japan.

Antimicrobial susceptibility test

All *Salmonella* isolates were inoculated into Mueller–Hinton broth (Oxoid) and were incubated overnight at 37°C. The turbidity of the suspensions was adjusted to a 0.5 McFarland standard and streaked onto Mueller–Hinton agar (Oxoid) plates. Antimicrobial disks were added on the plates and they were incubated aerobically at 37°C for 16-18 h were screened for susceptibility against 8 antibiotics by disc diffusion method according to Abd El-Razik *et al*., 2017 and assigned as sensitive, intermediate and resistant according to the recommendations of The Clinical and Laboratory Standard Institute (CLSI, 2017). The antibiotics used for the susceptibility testing are considered by the WHO as 'the most important drugs' still in use in healthcare settings (WHO, 2017). This included: Penicillins: ampicillin (10 µg); Phenicols: chloramphenicol (30 µg); Fluoroquinolones: ciprofloxacin (5 µg); Aminoglycosides: gentamycin (10 µg); Macrolide: erythromycin (15 µg); Tetracyclines: tetracycline (30 µg) Folate pathway inhibitor: sulfamethoxazole -trimethoprim (23.75-1.25 µg); Cephem: cephalothin (30 µg).

Detection of biofilm-producing genes in *Salmonella*

Genomic DNA extraction and PCR assay

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℃ 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 100 µl of elution buffer provided in the kit. The primers which were used supplied from Metabion (Germany) are listed in Table 1.

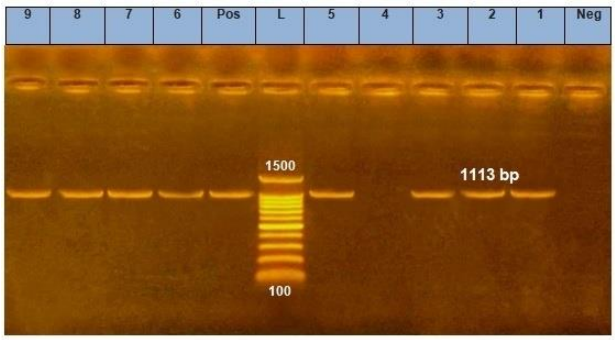

Photo 1: *adrA*gene; Lane1-2 *S. Entertidis*, Lane3-4 *S. Hadar*, Lane5-6 *S. Kentucky*, Lane7-8 *S.Typhimurium*, Lane 9 *S. Heidelberg*, Neg (negative control) and Pos (positive control).

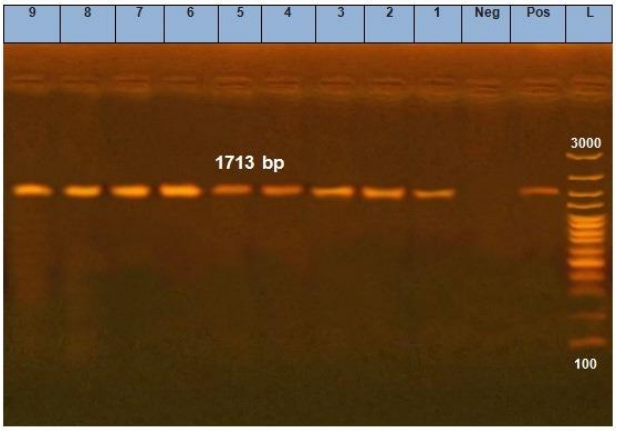

Photo 2: gcpA gene; Lane 1-2 *S. Entertidis*, Lane 3-4 *S. Hadar*, Lane 5-6 *S. Kentucky*, Lane 7-8 *S.Typhimurium*, Lane 9 *S. Heidelberg*, Neg (negative control) and Pos (positive control).

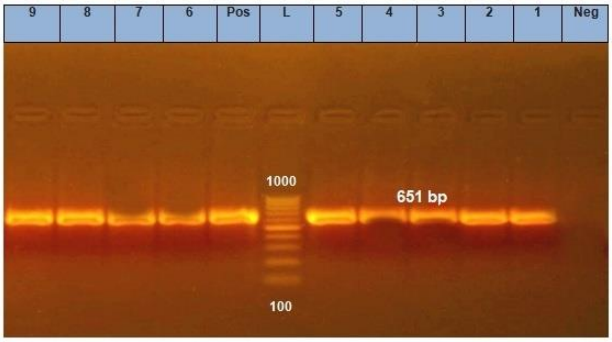

Photo 3: *csgD* gene. Lane1-2 *S. Entertidis*, Lane 3-4 *S. Hadar*, Lane 5-6 *S.Kentucky*, Lane 7-8 *S. Typhimurium*, Lane 9 *S. Heidelberg*, Neg (negative control) and Pos (positive control).

Primers were utilized in a 25- µl reaction containing 12.5 µl of DreamTaq Green PCR Master Mix (2X) (Thermo Scientific), 1 µl of each primer of 20 pmol concentration, 5.5 µl of water, and 5 µl of DNA template. The reaction was performed in an Applied biosystem 2720 thermal cycler. The products of PCR were separated by electrophoresis on 1% agarose gel (Applichem, Germany, GmbH) in 1x TBE buffer at room temperature using gradients of 5V/cm. For gel analysis, 20 µl of the PCR products were loaded in each gel slot. Gelpilot 100 bp plus DNA Ladder (Qiagen, Germany, GmbH) and Generuler 100 bp plus ladder (Fermentas, Thermo Scientific,

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
AdrA	ATGTTCCCAAAAATAATGAA	1113	94˚C	94˚C	50˚C	72˚C	72˚C	Bhowmick
	TCATGCCGCCACTTCGGTGC		5 min.	30 sec.	1 min.	1 min.	10 min.	*et al*., 2011
GcpA	CTATTTCTTTTCCCGCTCCT	1713	94˚C	94˚C	57˚C	72˚C	72˚C	
	GTGCCGCACGAAACACTGTT		5 min.	30 sec.	1 min.	1 min.	10 min.	
csgD	TTACCGCCTGAGATTATCGT	651	94˚C	94˚C	50˚C	72˚C	72˚C	
			5 min.	30 sec.	40 sec.	45 sec.	10 min.	

Germany) and Genedirex 100 bp DNA ladder H3 RTU, Cat. No. DM003-R500 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.

RESULTS

The results of serological identification of 9 *Salmonella* spp were illustrated in Table 2.

Table 2: *Salmonella* spp identification according to serological test.

Sample	No of strain	Serotype
Broiler internal organs	2	*S.Entertidis*
	2	*S.Hadar*
	2	*S.Kentucky*
	2	*S.Typhimurium*
	1	*S. Heidelberg*

Phenotypic antimicrobial resistance patterns

All *Salmonella* isolates were tested for their susceptibility to 8 antibiotics representing 8 different classes (Table 3). High level of antibiotic resistance was detected in all *Salmonella* isolates evaluated. All 9 isolates were resistant to ampicillin, erythromycin and cephalothin with percentage (100%), followed by tetracycline (44.4%), gentamycin (22.2%), ciprofloxacin and sulfamethoxazole -trimethoprim (11.1%) respectively as mentioned in (Table 3). In addition to that, the isolates showed intermediate-resistant to ciprofloxacin (55.5%) followed by gentamycin (22.2%) while all *Salmonella* isolates were susceptible to chloramphenicol (100%) and the percentage of susceptibility of the *salmonella* isolates began to decrease to be detected in sulfamethoxazole – trimethoprim (88.8%), gentamycin (55.5%), tetracycline (55.5 %) and ciprofloxacin (33.3%) respectively.

According to Table 4, the results reported that *adrA, gcpA and csgD* genes were detected with an incidence (8/9) 88.8%, (9/9)100% and (9/9)100% respectively.

DISCUSSION

Antibiotic resistance is frequently accompanied with infection and will be related to virulence for example biofilm-producing microorganisms or intracellular infections (Seral *et al.*, 2003 and Patel, 2005) and the direct involvement of efflux pumps is one of the common characteristics to virulence and resistance (Barbosa and Levy, 2000).According to antimicrobial susceptibility test results, there is a high level of antibiotic resistance in all salmonella isolates. All 9 isolates were resistant to ampicillin, erythromycin and cephalothin with percentage (100%), followed by tetracycline (44.4 %), gentamycin (22.2%), ciprofloxacin and sulfamethoxazole - trimethoprim (11.1%) respectively, all Salmonella isolates were susceptible to chloramphenicol (100%). This finding was completely supported by (Islam *et al*., 2016) who stated that, there is multidrug resistance was detected in case of apparently healthy and diarrheic broiler's samples, the results showed that salmonella strains were resistant to penicillin-G, erythromycin and ampicillin with an incidence 100%. (Elgohary *et al*., 2017) reported that sensitivity of salmonella *spp* to ciprofloxacin, gentamicin, trimethoprim were 56.3%, 50% and 18.8% respectively, all isolates were resistant to penicillin, this finding is nearly agreed with our results. (Leonal Rabins *et al*., 2018) isolated Salmonella from coriander leaves, the authors found that all the isolates were showing complete resistant to ciprofloxacin, chloramphenicol, gentamicin and cephalexin, this finding disagreed with our results. The current study stated that overuse of antibiotics as treatment or prophylaxis is the main cause of increase the incidence of antimicrobial resistance.

Biofilm is considered as a major virulence factor innumerous bacterial species, including *Salmonella spp*., being one of the significant explanations of chronic infections and environmental persistence (Seixas *et al*., 2014). The variations of biofilm formation-based on the diversity between *Salmonella* species as *S. Typhimurium* strains are considered as strong biofilms producers under different environmental conditions (Beshiru *et al*., 2018). Our study reported that all *Salmonella* strains (*S. Entertidis, S. Hadar, S. Kentucky, S. Typhimurium, S. Heidelberg*) carried biofilm genes approximately. The current study showed that *adrA, gcpA and csgD* genes were detected with an incidence (8/9) 88.8%, (9/9)100% and (9/9)100% respectively. This result somewhat agreed with (Hawash *et al*.,2017) who make surveillance on *salmonella* species in Egypt poultry farms, the authors stated that all samples showed positive PCR result for *csgD* gene and showed negative for *adrA* and *gcpA* genes. On the other side, our results fully agreed with (Seixas *et al*., 2014) who reported that, out of the 133 *Salmonella* isolates, which collected from environmental and animal origins all (100%) carried *adrA* and *csgD* genes, and 129 isolates (97.0%) were positive for *gcpA*, also (Abd El-basit *et al*., 2019) found that by using PCR all *S. enterica* strains had *adrA, csgD* and *gcpA* genes and that biofilm formation were found in all *Salmonella* isolates causing the increase of tolerance for antimicrobial agents and disinfectant, leading to hardness in the curing of ailment that cause a lot of problems in food industry as it turns to be a continuous reason of contamination.

Table 3: Determination of phenotypic antibiotic resistance profiles of *Salmonella* isolates recovered from avian sources.

	Salmonella spp. Antibiotics							
	Penicillins	Macrolides	Tetracyclines	Amino-glycosides	Fluoro-quinolones	Cephem	Phenicols	Folate pathway inhibitor
	Ampicillin (10 µg)	Erythromycin (15 µg)	Tetracycline (30 µg)	Gentamycin (10 µg)	Ciprofloxacin (5 µg)	Cephalothin (30 µg)	Chloram-phenicol (30 µg)	Sulfamethoxazole -trimethoprim (23.75-1.25 µg)
S. Heidelberg	R	R	R	S	I	R	S	S
S.Entertidis	R	R	R	R	I	R	S	S
S.Entertidis	R	R	R	S	I	R	S	S
S.Hadar	R	R	S	I	R	R	S	R
S.Hadar	R	R	S	S	S	R	S	S
S.Typhimurium	R	R	S	R	S	R	S	S
S.Typhimurium	R	R	S	S	I	R	S	S
S.Kentucky	R	R	R	I	I	R	S	S
S.Kentucky	R	R	S	S	S	R	S	S

Table 4: PCR results of biofilm genes (*adrA,gcpA and csgD*).

	Results		
Serotype	*adrA*	*gcpA*	*csgD*
S.Entertidis	+	+	+
S.Entertidis	+	+	+
S.Hadar	+	+	+
S.Hadar	-	+	+
S.Kentucky	+	+	+
S.Kentucky	+	+	+
S.Typhimurium	+	+	+
S.Typhimurium	+	+	+
S. Heidelberg	+	+	+

Biofilms assume a significant role to secure bacteria against harm by various natural factors, therefore, the bacteria can exhibited resistance to antibiotics that is hard to supersede, this finding was supported by (Farahani *et al*., 2018) who mentioned that, there was a strong relationship between the biofilm formation and the antibiotic resistance to colistin, ceftazidime, chloramphenicol, gentamicin, trimethoprim, penicillin, and trimethoprim-sulfamethoxazole. On the other side, (Ghasemmahdi *et al*., 2015) mentioned that nearly all *S. Typhimurium* isolates showed a high multiple antibiotic resistant with low biofilm forming capabilities that intended low association between biofilm formation and antibiotic resistance.

Conclusions

Poor sanitary conditions and low economic resources in developing countries are the main sources of biofilm formation on equipment and tools that increase the hazard factors causing pollution during food preparation and finally, increasing the frequency of antibiotic resistance. Use of antibiofilm approaches leads to lowering the rate at which antibiotic resistance arises.

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