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Molecular Characterization of Biofilm Producing Genes in Salmonellae Isolated from Chicken

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ABSTRACT

Salmonella enterica considered one of the most important food-borne pathogen. Biofilm formation considered one of the main problems related to *S. enterica*. In this study, biofilm formation, colony morphotype, cellulose and curli production genes of 19 *Salmonella* isolates were tested. The results showed that 85% of isolates produced strong biofilm and 15% of isolates produced moderate biofilm on polystyrene plate with 1/20 diluted TSB. Different colony morphotypes expressed saw, sbam, and rdar morphotype when cultivated on LB containing Congo red for monitoring cellulose and curli production. All *S. enterica* strains possess *adrA*, *csgD* and *gcpA* genes using PCR. Thus in this study all *Salmonella* isolates formed biofilm so they give increased tolerance for antimicrobial agents and disinfectant, which results in difficulty in the treatment of diseases and causing many problems in food industry as it becomes a persistent of source of contamination.

Introduction

Salmonella is a gram-negative, rod shaped, facultative anaerobic Enterobacteriaceae, it comprises more than 2500 different serovars. Salmonellosis is one of the leading food-borne diseases and a major public health threat worldwide. Localization of bacteria in the environment and in the infected host obtained by the formation of exopolymer matrix known as biofilms (Grantcharova *et al*., 2010). Microbial biofilm described as an accumulation of cells, mixed species or mono-species, which is attached to a surface and or to each other. Biofilm cells produce proteinaceous substances that protect bacteria from negative environmental condition (Donlan and Costerton, 2002), and become highly resistant to antibiotic (Brown *et al*., 1988).

Salmonella produce biofilms on biotic and abiotic surfaces like plastic, rubber, and stainless steel (Joseph *et al*., 2001; Solano *et al*., 2002). Cellulose considered one of the main components of a biofilm, and the other components as curli are responsible for different morphotypes showed by *S. enterica* on congo red agar. The characteristic rdar (red, dry and rough) morphotype characterized by cellulose and curli fimbriae production (Chia *et al*., 2011). This morphotype is closely related to the *csgD* and *adrA* genes, which are responsible for the regulation and expression of cellulose, respectively (Fabrega and Vila, 2013). Biofilm bacteria become more resistant to the effect of antimicrobial agent and disinfectant (Joseph *et al*., 2001; Scher *et al*., 2005; Wong *et al*., 2010). Biofilm formed on different food contact surfaces can become a persistent source of contamination leading to foodborne diseases or food spoilage (Shi and Zhu 2009; Vestby *et al*., 2009). Biofilm formation by *S. enterica* is an issue of concern in the food industry as *Salmonella* in biofilms are more protected against antibiotics, disinfectants and environmental stresses than the planktonic cells, which leads to failure in treatment and difficulty of eradication. Thus, this study aimed to detect the ability of *Salmonella* isolates to form biofilm and the presence of genes responsible for producing biofilm.

Materials and methods

Strain identification

A total of 19 *Salmonella* strains were isolated from sam-

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ples that collected from broiler chickens in poultry farms in different localities in Luxor city, Egypt during 2014. Chickens showed suspected signs of salmonellosis, the age of these chickens ranging from 5 to 15 days old.

Nine different serotypes were detected (*S. kentucky*, *S. agona*, *S. bovismorbificans*, *S. newport*, *S. vellore*, *S. sandiego*, *S. powell*, *S. nigeria* and *S. virchow*), the prevalence of these serotypes was 31.6%, 15.8%, 15.8%, 10.5%, 5.26%, 5.26%, 5.26%, 5.26%, 5.26% respectively.

Isolation of Salmonella species

Salmonella species were isolated and identified biochemically according to ISO-6579:2002 standards.

Serological identification of Salmonella

Typing of *Salmonella* isolates was performed according to Kauffman-White scheme (Kauffmann, 1974).

Inoculation of Salmonella isolates

For inoculation, the strains were transferred from the stock cultures into tryptic soya broth (Difco, MI, USA) and incubated overnight at 37˚C (Stepanovic *et al*., 2004). The incubated cultures were used for inoculation into different media poured into the wells of plastic microplates (Greiner Bio-One, Germany) for later quantification of biofilm production.

Microtitre plate assay

The quantification of biofilm formation by *Salmonella* isolates was assessed using microtitre plate assay as described by Nair *et al*. (2015). Wells of polystyrene microplate filled with 180 µl of 1/20 diluted tryptic soya broth, 20 µl of cultures of each isolate incubated in TSB dispensed into the wells of polystyrene microplate in triplicate. Negative control wells in triplicate contained 200 µl of broth only. The inoculated plates were incubated aerobically at 37°C for 72 h then the contents of the plates were poured off and the wells thoroughly washed thrice with PBS (pH 7.2). Attached bacterial cells were stained with 200 µl of 0.5% (w/v) crystal violet (Fluka, UK) per well for 10 min. After staining, the plates were washed three times with 300 µl/well of sterile distilled water and allowed air dried, the stained adherent bacterial cells from each well resolved with 250 µL 33% of glacial acetic acid. The optical density (O.D.) of each well was measured at 600 nm using an automated microplate reader (Tecan sunrise, Jencons, UK).

The cut-off O.D. (ODc) was defined as three standard deviations above the mean O.D. of the negative control. Thus based on ODc, the *Salmonella* isolates were classified into four categories: i. Non biofilm producers: O.D. of test isolates ≤ ODc, ii. Weak biofilm producers: O.D. of test isolate ≤ (2×ODc), iii. Moderate biofilm producers: O.D. of test isolate ≤ (4×ODc), iv. Strong biofilm producers: O.D. of test isolate > (4×ODc).

Congo red agar plating

It was done according to Romling *et al*. (2003) as bacterial culture plated on LB agar without salt supplemented with 40 µg/ml Congo Red (AppliChem, USA) and 20 µg/ml Coomassie brilliant blue (AppliChem, USA). The colony morphology was determined after incubation at 28°C for 48h.

Detection of csgD, adrA and gcpA genes by polymerase chain reaction

Nineteen *Salmonella* isolates were extracted according to QIAamp DNA mini kit instructions (Qiagen, Germany). Specific PCR primers were used as listed in Table 1, and were supplied from Metabion (Germany). Each PCR reaction was carried out in a 25 µL master mix containing 12.5 µL of Emerald Amp Max PCR Master Mix (Takara, Japan), 1 µl of 20 pmol conc. of each primer, 4.5 µL of water and 6 µL of template DNA. Amplification was conducted in Biometra T3 thermal cyclers. The PCR conditions included an initial denaturation of 94°C for 5 min, followed by 30 cycles of denaturation at 94°C for 1 min, primer annealing at 50°C for *csgD* and *adrA* genes and 57°C for *gcpA* gene for 1 min, extension at 72°C for 1 min and a final delay at 72°C for 5 min (Bhowmick *et al*., 2011).

This work and the used procedures were performed according to the ethical standards of Animal Health Research Institute and Veterinary authorities in Luxor province, Egypt.

Results

Biofilm formation on polystyrene plates

Results demonstrated that all *Salmonella* isolates produced biofilm when cultivated in the appropriate medium 1/20 diluted tryptic soya broth. Among 19 *Salmonella* isolates 16 isolates (85%) produced strong biofilm and the other three isolates (15%) produced moderate biofilm as shown in Table 2.

Congo red plate

Three different colony morphotypes were detected on Congo red (CR) agar plates (rdar, saw and sbam). rdar (red, dry and rough morphotype) indicating curli and cellulose production, saw (smooth and white morphotype) indicating a lack of both curli and cellulose production finally sbam (smooth brown and mucoid colony) indicating a lack of cellulose synthesis but overproduced capsular polysaccharide.
The prevalence of sbam, saw and rdar morphotypes was 52.6%, 31.5%, and 15.7%, respectively.

PCR Detection of csgD, adrA, and gcpA

All *Salmonella* isolates analyzed and the results detected that 100 % of isolates were positive for the *csgD* (Fig. 1), *adrA* (Fig. 2), and *gcpA* (Fig. 3) genes.

Table 1. Oligonucleotide primers sequences used to detect specific genes

Gene	Primer Sequence (5`-3`)	Length of amplified product	Reference
adrA	ATGTTCCCAAAAATAATGAA TCATGCCGCCACTTCGGTGC	1113 bp	Bhowmick *et al*. (2011)
gcpA	CTATTTCTTTTCCCGCTCCT GTGCCGCACGAAACACTGTT	1713 bp	
csgD	TTACCGCCTGAGATTATCGT ATGTTTAATGAAGTCCATAG	651 bp	

Table 2. Biofilm formation by different *Salmonella* isolates.

Salmonella serotypes	Biofilm formation	Colony morphology	PCR detection
			csgD, *adrA*, *gcpA*
S. kentucky	Strong	rdar[a]	+
S. kentucky	Strong	rdar	+
S. kentucky	Strong	saw[b]	+
S. kentucky	Strong	rdar	+
S. kentucky	Strong	sbam[c]	+
S. kentucky	Strong	sbam	+
S. agona	Strong	sbam	+
S. agona	Strong	sbam	+
S. agona	Strong	sbam	+
S. bovismorbificans	Strong	saw	+
S. bovismorbificans	Strong	sbam	+
S. bovismorbificans	Strong	saw	+
S. sandiego	Strong	sbam	+
S. nigeria	Strong	saw	+
S. newport	Moderate	sbam	+
S. newport	Strong	sbam	+
S. vellore	Moderate	sbam	+
S. virchow	Strong	saw	+
S. powell	Moderate	saw	+

[a]rdar: red dry and rough morphotype; [b]saw: smooth and white morphotype; [c]sbam: smooth brown and mucoid colony.

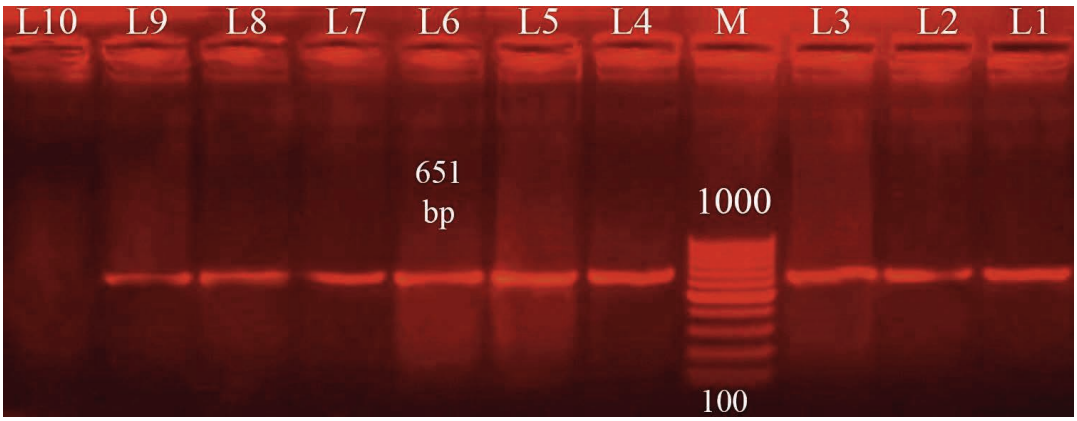

Fig. 1. PCR result for *csgD* (651 bp) gene. M: 100-1000 bp DNA ladder. Lane 1, 2, 3, 5, 6, 7, 8, 9: *Salmonella* isolates positive for detection of *csgD* gene. Lane 4: Positive control sample. Lane 10: negative control sample.

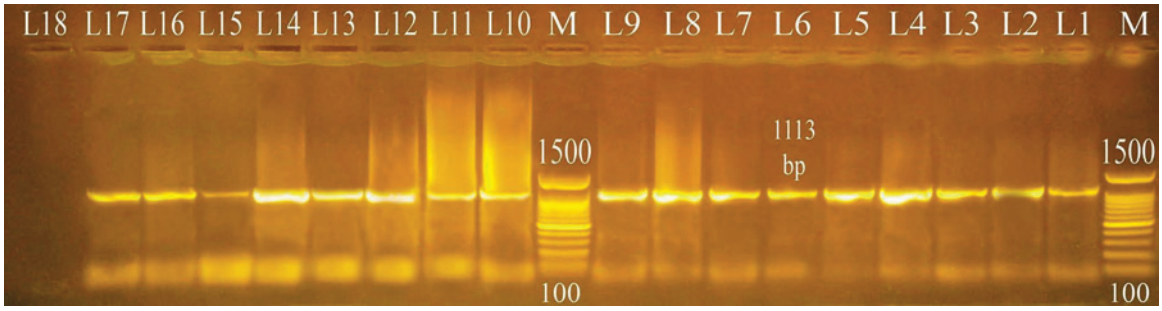

Fig. 2. PCR result for *adrA* gene (1113 bp) M: 100-1500 bp DNA ladder. Lane 1: Positive control sample. Lane 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17: *Salmonella* isolates positive for detection of *adrA* gene. Lane 18: Negative control sample.

Discussion

Salmonella enterica subsp. enterica is one of the main food-borne pathogens, which responsible for 88.715 cases of salmonellosis in 2014 in the European Union (EFSA, 2015), *Salmonella* spp. considered important pathogenic bacteria, which could be transmitted by food. *S. enterica* possess recurrent lifestyle that implicated colonization of host with survival in a specific environment. This implies that this pathogen can adapt rapidly from an anaerobic to an aerobic metabolism in order to survive (Encheva *et al.*, 2009). The ability of *S. enterica* to form biofilms is an issue of concern in food industry, biofilm formation by *Salmonella* considered important virulence factor. In biofilm cells were more protected against harsh environmental factor, disinfectants, antibiotics and immune system of the host than the planktonic cells (Jensen *et al.*, 2010).

Different studies have demonstrated the ability of *Salmonella* to adhere and form biofilms on a different food contact surfaces, such as metal, plastic and rubber (Joseph *et al.*, 2001;

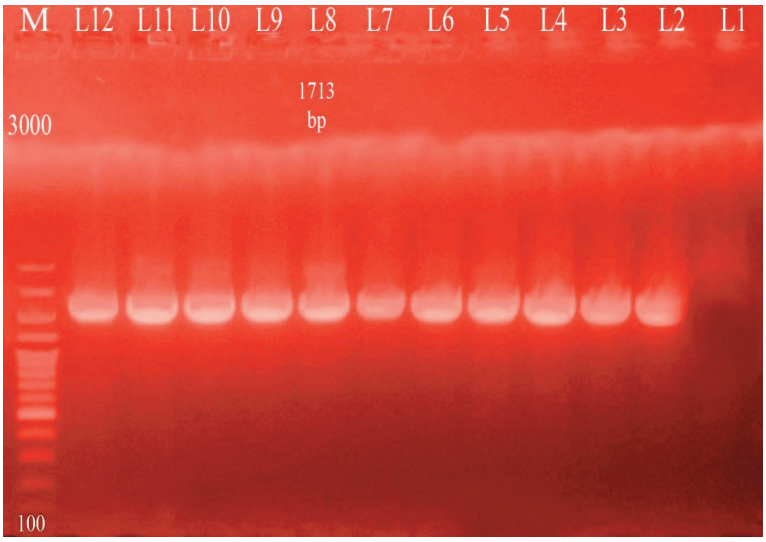

Fig. 3. PCR result for *gcpA* gene (1713 bp). M: 100-3000 bp DNA ladder.Lane 1: Negative control sample. Lane 2,3,4,5,6,7,8,9,10,11. *Salmonella* isolates positive for detection of *gcpA* gene. Lane 12: Positive control sample.

Stepanovic *et al*., 2004). Biofilm formed in food processing environments had special importance as it became the source of the chronic microbial contamination, which may lead to food spoilage and disease transmission. A biofilm defined as a community of bacterial cells that adhere to a surface and embedded in a self-produced polymeric matrix (Costerton *et al*., 1999). In the present study, 19 *Salmonella* isolates investigated for production of biofilm on polystyrene microtiter plate, the results demonstrated that all *Salmonella* isolates produced biofilm, which in agreement with the reports about *Salmonella* biofilm on plastic surface obtained by Solomon *et al*. (2005) and Milanov *et al*. (2017) as their studies recorded the same results. Lu *et al*. (2011) found that 39 isolates from 62 *S. enterica* serovar Pullorum isolates have the ability to produce biofilm, and Malcova *et al*. (2008) recorded that 80% of *Salmonella* typhimurium isolates formed biofilm. In this study 85% of isolates were strong biofilm former, while 15% of isolates were moderate biofilm former, while Silva *et al*. (2014) evaluated the biofilm formation of *Salmonella* enteritidis on polystyrene and recorded that 25% of isolates were strong biofilm, 35% moderate biofilm, 35% weak biofilm and 10% non-biofilm forming. The differences in biofilm formation could be attributed to strain variations (Chelvam *et al*., 2014). Nair *et al*. (2015) found that the majority of isolates of *Salmonella* formed biofilm (85%), among 40 isolates; 27 isolate were weak biofilm producers and 7 isolates were moderate biofilm producers. Also, other previous studies reported that most of the *Salmonella* isolates were weak biofilm formers (Diez-Garcia *et al*., 2012; Ghasemmahdi *et al*., 2015), while moderate biofilm *Salmonella* producers were reported by Agarwal *et al*. (2011) and Naeem *et al*. (2014).

Poultry is considered a major reservoir for *S. kentucky* (Weill *et al*., 2006), which then can be transmitted to human from both poultry meat and eggs. Our results indicated that all *Salmonella Kentucky* isolates form strong biofilm, also Turki *et al*. (2012) examined 57 *Salmonella Kentucky* strains isolated from different sources, and the majority of these strains were able to form a biofilm, especially for environmental and animal source isolates. So, the biofilm tends to be a normal and favorable capability in the life of *S. kentucky* in the environment. *S. kentucky* was linked with human diseases (81 human cases in 2005), although widespread in the food supply chain, particularly in poultry, in which it has been the most frequent serotype encountered (CDC, 2006; Joerger *et al*., 2009).

Salmonella enterica forms biofilm on biotic and a biotic surface, this *Salmonella* produces an extracellular matrix with curli and cellulose. Curli are amyloid fibers, which play a role in adhesion to surface, cell aggregation, colonization and biofilm formation (Steenackers *et al*., 2012). Cellulose, one of the main components of a biofilm which facilitates cell attachment. The characteristic rdar (red, dry, and rough) morphotype, characterized by cellulose and curli fimbriae production, was well studied in *S. enterica* and allowed it to persist in nutrient-limited environments (Chia *et al*., 2011).

Bacteria produce both curli and cellulose display a red, dry and rough colony morphology (rdar) on Congo red agar plates. Deficiency in curli and cellulose synthesis causes a smooth and white (saw) colony appearance, brown (bdar) colonies obtained by a defect in cellulose synthesis as curli give these brown colony, while the deficiency in curli lead to a pink (pdar) morphotype (Romling, 2005).

The result of Congo red in this study indicated that *Salmonella* spp. showed different morphotypes as three isolates showed rdar, another six showed saw and ten isolates showed sbam morphotype. Similar studies obtained different morphotypes as Malcova *et al*. (2008) detected different colony morphotypes among 94 strains, 17 strains described saw morphotype, in 10 strains the bdar morphotype was identified and the rdar morphotype was found in 62 strains. Turki *et al*. (2012) detected the colony morphology for 57 *S. kentucky* isolates, where five different morphotypes were detected; saw (24), rdar (22), sbam (7), bdar (1) and srad (3). Lu *et al*. (2011) identified that non biofilm-forming showed nearly white colonies, while biofilm producers showed pink to red colonies. Two morphotype detected in the study of Chia *et al*. (2011) as all strains display the RDAR morphology except one strain that was a natural and typical BDAR morphotype of this serovar.

Lamas *et al*. (2016a) found that all *S. enterica* expressed the rdar morphotype in aerobiosis condition and this result disagreed with the present study. *S. enterica* is in aerobiosis conditions when the bacteria survive outside the host. In these environmental conditions, biofilm formation is an important resource for survival (Fabrega and Vila, 2013). Also Milanov *et al*. (2017) observed the same result as all isolates of S. tennessee formed the characteristic "rdar" colonies on Congo Red agar. Solano *et al*. (2002) obtained result near to our result as 93% of *Salmonella* isolates showed rdar morphotype on CR.

Although in this study, all serotypes formed strong and moderate biofilm not all serotypes formed rdar morphotype and mostly of them formed saw and sbam morphotype so the production of curli and cellulose not the only parameter for biofilm formation (Lamas *et al*., 2016a).

Biofilm formation in *S. enterica* was regulated by different genes. The *csgD* gene share into biofilm production by its important role in coinciding the expression of several determinants of this process. The *adrA* gene was essential for biofilm formation by regulating cellulose synthesis through c-di-GMP, which mediates the post transcriptional activation of cellulose biosynthesis (Steenackers *et al*., 2012).

Adhesion of bacteria on surfaces activated the expression of biofilm genes, which are responsible for the fimbriae and cellulose expression in *Salmonella*. Cellulose and curli were the major constituents of the biofilm matrix; the curli synthesis process is encoded by two operons, *csgDEFG* and *csgBA*. *CsgA*, which is the major curli subunit and CsgB minor subunit encoded by csgBA, *csgD* is necessary for the transcription *csgBA*.

CsgD also regulates the production of cellulose and requires the expression of *adrA* gene that activates cellulose synthesis at the post-transcriptional level (Chapman *et al*., 2002). GcpA (GGDEF domain containing protein A coded by *gcpA*) protein plays a vital role in inducing biofilm by *S. Typhimurium* at low nutrient conditions.

In this study, we detected the presence of the three genes (*adrA*, *csgD* and *gcpA*) involved in biofilm biosynthesis using PCR and all isolates expressed the three genes. Olivera *et al*. (2014) agreed with our result as they detected the presence of *adrA* gene in 112 *Salmonella* isolates and all of samples positive for *adrA* gene, which in accordance with our result. Although we have observed a strong association between agfD and *adrA* genes and the production of biofilm, just a limited proportion of strains were able to produce the characteristic rdar morphology on LB culture medium. The lower percentage of rdar colonies at 28°C (2.3%) could be explained by the observation that thin aggregative fimbriae production in *Salmonella* spp. was regulated by environmental conditions that play a role on the *agfD* promoter for triggering the cascade of biofilm production (Romling *et al*., 1998). In addition, according to Gerstel and Romling (2003), oxygen and pH variation could also interfere on biofilm formation by *Salmonella Typhimurium*, and directly influence the expression of rdar morphology. Another possibility is that the rdar morphology test is not sensitive enough to detect weak biofilm-producer strains. Also Zeich *et al*. (2016) agreed with these study assess the presence of the most important to *Salmonella* biofilm formation genes *adrA* and *csgD* in these strains and detected both genes in all examined strains.

Seixas *et al*. (2014) agreed with this study in the presence of *adrA* and *csgD* genes 100% of isolates positive for *adrA* and *csgD*, and disagreed in detection of *gcpA* 97.0 % of isolates were positive for *gcpA*. Bhowmick *et al*. (2011) detected the genes *csgD*, *adrA* and *gcpA* in all examined isolates, with the exception of SW13 in which *adrA* and *gcpA* were negative (Gene expression of *gcpA*, *adrA* and *csgD* was studied by real-time PCR). Lamas *et al*. (2016b) indicated that among the tested strains, 68.49% (50 of 73) were positive for *csgD*, 80.82% (59 of 73) were positive for *adrA*, and 41.10% (30 of 73) were positive for gpcA.

The morphotype rdar (red, dry, and rough) was closely related to the *csgD* and *adrA* genes, which were responsible for the regulation and expression of cellulose, respectively (Fabrega and Vila, 2013), all *Salmonella* strains were positive for presence of *csgD*, *adrA* and *gcpA* genes but not all of them expressed rdar morphotype, which indicated that the expression of this morphotype was dependent on certain environmental conditions.

Conclusion

The characterization of biofilm formation for different *Salmonella* isolates revealed that all isolates formed biofilm and different colony morphotype. However, *adrA*, *csgD* and *gcpA* presented in all isolates not all of them expressed the RDAR morphotype. Finally, Biofilm formation allowed the *Salmonella* to survive within the host and the environment and become persistent source of contamination.

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Conflict of interests

The authors have declared that no competing interests exist.

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