Research Article

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Phenotyping and Genotyping of *Salmonella enterica* as Biofilm Producer Isolated from Diarrheal Animal and Human

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Abstract : This study aimed to find out how different stress conditions (like temperature and pH) affect Salmonella enterica biofilm formation. This was done by looking at the phenotypic and genotypic features of isolates. 12 Salmonella enterica Isolate from animals, and 13 Salmonella enterica Isolate from people were used. S. enterica isolates were grown in tryptic soy broth (TSB) at (37°C, 25°C, and 42°C), and at pH levels (7, 5, and 9). The results revealed that the percentage was 52% in the standard conditions (temperature 37ºC and pH 7) while, in another condition, observed in the same temperature (37 °C) but with pH differences (pH 5, pH 9). *S. enterica*, did not produce biofilm. As for the stability pH in the, pH 7 with a change in, the temperature at 25°C percentage, biofilm produce (44%) while in 42 °C (64%). The detection rates of genes, biofilm-related PCR was used to find BapA and CsgD, were 100%. Although the biofilm formation of the phenotype did not give 100% results, the genotype gave 100%, which indicates that the gene is present but not expressed. Based on the findings in this study provided valuable information on the biofilm formation of Salmonella isolated from animals and humans.

Keywords: *Salmonella*, Biofilm, *BapA* gene, *CsgD* gene.

Introduction

Salmonella's ability to create biofilm varies and depends on the isolates' serotypes or origins (1-3). Salmonella species are reported to produce biofilms on both biotic (epithelial cells and gallstones) and abiotic (plastic, glass, and metal) surfaces (4).

Numerous variables, including species, surface, available nutrients, and other environmental circumstances, influence the growth and structure of a biofilm (5-7). Intercellular interactions within and between distinct bacterial species are thought to play crucial roles in biofilm, development and antibiotic resistance (8). Numerous prior research aimed at elucidating the stress tolerance, survival, and mechanisms of Salmonella, related these processes to the capacity of these bacteria to cling to various abiotic and biotic surfaces and form biofilms (9). Biofilm is, composed of bacteria that reside as planktonic cells in bulk solution and sessile cells that form a unit to adhere to a surface (10). *Salmonella enterica* serovars consist of *Salmonella typhimurium* and *Salmonella enteritidis,* which are responsible, for the bulk, of salmonellosis, with a considerable rise in their ability to build biofilm (11,12). Biofilm formation is also influenced by environmental factors such as temperature and pH, which are always dynamic and vary enormously in the modern food-producing environment (13). Curli only and cellulose production has been described to be involved in the *S. enterica* biofilm formation process so far, (14) Creates a biofilm with a matrix made primarily of curli fimbriae and cellulose. The discovery of BapA, a major cell-surface protein needed for salmonella, biofilm development (15). The CsgD gene is a component of the curli,fimbria-encoding csgBAC-csgDEFG complex. Fimbriae are essential structures for the biofilm formation process, as they are crucial for promoting the first cell surface attachment (16,17). Salmonella's biofilm production is controlled by, which acts as a master transcriptional regulator. It acts by stimulating the manufacture of matrix-specific, protein (17,18). The study's goal is to determine the effect of many different environmental conditions on *S. enterica* ability to make biofilm.

Materials and methods

Bacterial isolates: The period of the study was extending started from December 2020 to September 2021. The total number of collected samples was 400, including source, 200 diarrheal samples for each from animals and humans. The samples examined for detection, *Salmonella enterica* ,in Al-Nasiriyah city in the south of Iraq. All the strains were proven to be *S. enterica* by conventional, biochemical and ApI-20E tests at the Department of Public Health, Laboratories of Technical, Institute, Southern Technical University. The purity of the strains was determined by culturing them on selective media (XLD and SSA). All the isolates were maintained at -80ºC in 25% glycerol (19).

Biofilm formation and quantification of *Salmonella* strains: Microtiter-plate, the test was used to detect the biofilm formation for all bacterial isolates, according to (19). Bacterial isolates (20 µl) overnight cultures were used to inoculate 96-microtiter wells plate containing 180 µl of Tryptic soy broth (TSB). The bacterial strain was inoculated, then incubated at 37°C,25ºC, and 42 ºC for 18hrs and different pH in media pH 5, pH 7, and pH9. Bacterial isolates (20 µl) overnight cultures were used to inoculate 96-microtitier, wells plate containing 180 µl of Tryptic soy broth (TSB). Cultures were

removed, and the wells were rinsed with phosphate buffer slain (PBS) (pH 7.2). Negative control wells contained the broth only. The plate's content was then poured off, and the wells were washed three times with 200 µl of sterile distilled water. The remaining attached bacteria were fixed with 200 µl of methanol per well, and after 15 min, microplates were emptied and air dried. The microplate was, stained with 200 µl per well of crystal violet used for Gram staining for 5 min. The optical density (O.D) value was measured at 570 nm by a plate reader (20).

Biofilm degree =Mean OD570 of tested bacteria- Mean OD570 of control the average OD570 nm of the absorbance.

PCR based screening of biofilm producing *Salmonella*: All of the *Salmonella* isolates previously shown to produce biofilms phenotypically were tested for specific genes associated with biofilm production. DNA extraction from bacteria was stored in broth using Bosphore® Bacterial DNA Extraction Spin Kit (Anatolia, Turkey). The method is based on the silica membrane column, a separation that involves the extraction of nucleic acids by removing and purifying the bacterial nucleic acids (19). Molecular biofilm detection is achieved by detecting BapA gene (21) and CsgD gene (22). by PCR using specific oligonucleotide, primers as described in Table 1.

Analysis of the data

Statistical analysis was done on the results of the analytical tests with the software IBM SPSS 22.

Table 1: Primers sequences used for genes amplification

Gene	Primer Sequences (5'-3')	Product Size(bp)	Reference
BapA	F CTACTCGCCGTGGTTTCG	858	(21)
	R CGGCATCATCGATAGCGG		
CsgD	F CGTGCGTCACCCTTCATG	760	(22)
	R CTCTTCGATGGCGTTATTTTC		

Results

The present study was conducted to determine the biofilm production, ability, and virulence of, *Salmonella* isolates recovered from diarrheal animals and humans isolated from the south of Iraq. A total of 25 *Salmonella* were recovered and identified, of which 12 (48%) were from animals, and 13 (52%) were from humans. Effect of incubation, temperature, and pH on biofilm formation: showed that the incubation temperature and pH significantly affected the formation of biofilm by *Salmonella enterica*, and pH value also significantly affected the ability to form a biofilm ($p > 0.05$). The formation of biofilm differed, in different conditions, where the percentage was 52% in the standard, conditions (temperature 37ºC and pH 7), ranging from weak (6), moderate (6), and strong (1). In contrast, in another condition,

observed at the same temperature (37ºC) but pH differences (pH 5, pH 9), *S. enterica* did not produce biofilm. As for the stability pH, the pH of 7 with a change, in temperature, in 25ºC the percentage of biofilm produced (44%) arranged between 10 isolates weak. One isolate was moderate while in 42ºC (64%), arranged between 10 isolates, weak while 4 isolates were moderate and 2 isolates strongly in Table (2). Figure (1): Biofilm strength percentage of the 25 isolates of *S. enterica* under different conditions.

Detection of Biofilm Formation by BapA and csgD genes

All DNA of S. enterica was, subjected to standardized PCR for detection of BapA (biofilm association protein) csgD gene (encodes curli fimbria). All S. enterica were positive and percentages of 100 % (n=25) for BapA gene and 100% (n=25) for csgD gene as in Figures (2, 3).

Table (2) Quantitatively detection outcomes of strength biofilm formation by MTCP of *S. enterica isolates* under different conditions

Environmental condition	Sources of isolate								P value
	Human (No.=13)				Animals (No.=12)				
	N /No/%	W	M	S	N/No/%	W	M	S	
pH 7/ Tem/ 37 ºC	6(46.15)	2(15.38)	4(30.76)	1(7.69)	6(50)	4(25)	2(16.66)	0(0)	0.513*
pH /5 Tem / 37℃	13(100)	0(0)	0(0)	0(0)	12(100)	0(0)	0(0)	0(0)	1*
pH /9 Tem/ 37℃	13(100)	0(0)	0(0)	0(0)	12(100)	0(0)	0(0)	0(0)	1*
pH /7.5 Tem/25℃	7(53.84)	6(46.15)	0(0)	0(0)	7(58.33)	4(25)	1(8.33)	0(0)	0.506*
pH /7.5 Tem/42℃	4(30.76)	4(30.76)	3(23.07)	2(15.38)	5(41.66)	6(50)	1(8.33)	0(0)	0.093*
P value	0**				0**				

*No Significant difference at P<0.05 ** Significant difference at P<0.05

N: negative, W: weak, M: moderate, S: Strong.

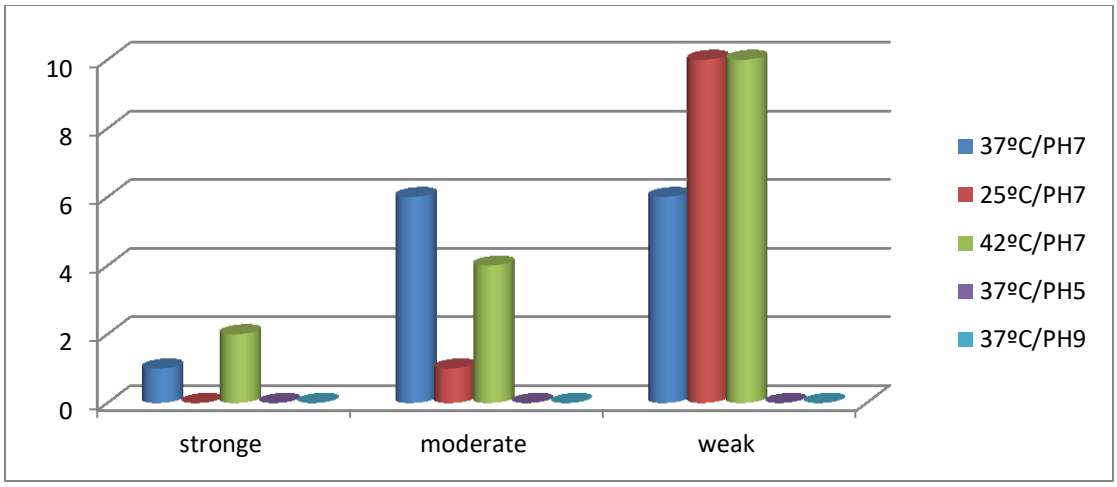

Figure (1): Biofilm strength percentage of the 25 isolates of *S. enterica* under different conditions.

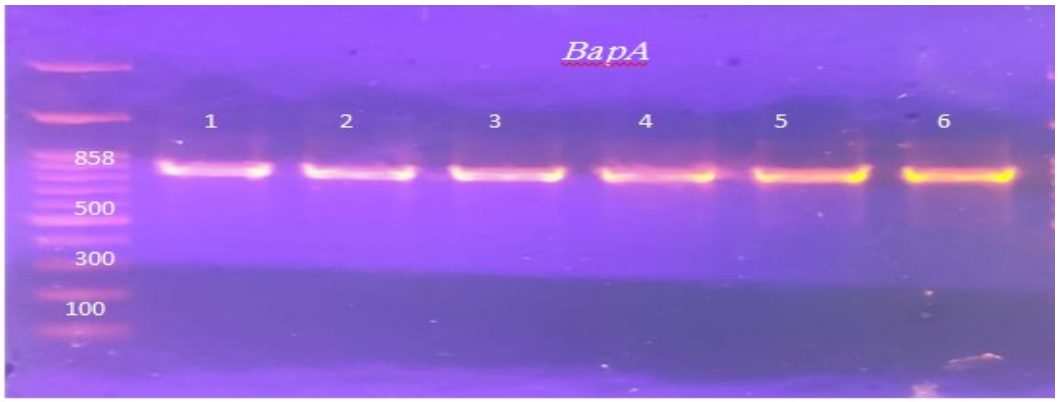

Figure (2): PCR products of the *BapA gene* of *S. enterica.*the size of the PCR product is 858 pb M: Marker DNA ladder (100-1500 bp)

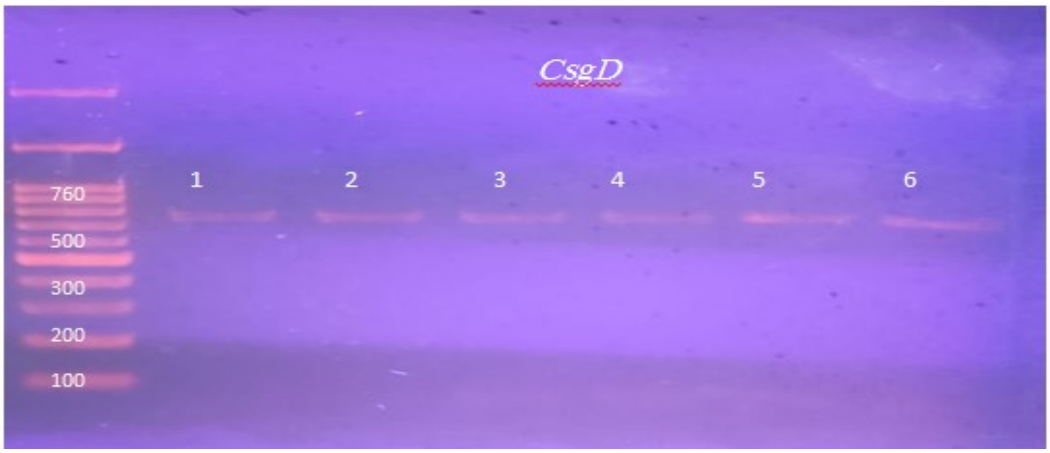

Figure (3): PCR products of the *CsgD gene* of *S. enterica.* The size of the PCR product is 760 pb M: Marker DNA ladder (100-1500bp)

Discussion

Biofilm is well-established as a major virulence factor in numerous bacterial species, including Salmonella spp., as well as, one of the primary causes of chronic infections and environmental persistence (4). The ability of different bacteria to form a biofilm has long been considered a key factor for survival and persistence in different environments (23,24). Different factors include pH, temperature and incubation, period influence, and the biofilm formation process (25). The present results appeared that all isolates were biofilm formation, and most of these isolates were 52% in the standard conditions (temperature 37ºC and pH). *S. enterica* did not produce biofilm at (37ºC) temperature, and pH differ (pH 5, pH 9), while at 25ºC percentage of biofilm was produced (44%) and at 42ºC (64%). (26) and (27) demonstrated that more than 30% of isolates were non-biofilm forming, contradicting (28) that 21.9% of isolates were non-biofilm producing. These differences result from different isolation sources and disagree with (29). Because of differences in the species of bacteria, (10) observed that the rate of biofilm formation increased with increasing temperature and pH. At the same time, the number of attached cells after 24 hr. decreased, with increasing temperature, and was not different between pH 6 and 7. A study by (21) Mentioned that all isolates produce biofilm because the serotype of Salmonella strains affected the ability of biofilm formation, which is consistent with previous reports (30,31). All tested, Salmonella strains produced biofilm at 25ºC and 37ºC (32). Many studies show that the same things happen when biofilms form (33,34). Also, (25) referred those optimum environmental conditions (temperature 37ºC, pH 7.0, and 0.25% glucose) exhibited strong biofilm formation on food and food contact surfaces and increased the virulence gene expression levels. The current study did not agree with what (35) found that 85% of Salmonella isolates could form biofilm. This study disagreed with (36) and found that 34.5% of the isolates were strong biofilm producers, while 59.6% and 5.7% of the isolates were moderate and weak biofilm producers. Another study (37) found that more *Salmonella enteritidis and typhimurium* isolate made weak biofilm at different temperatures. However, (30,38) found that they made strong biofilm. The explanation for the lack of biofilm formation at a high pH degree, according to (10), suggests that low pH conditions level slows

down the rate at which biofilms form by mainly affecting how bacteria stick together at first. Biofilm, association protein (BapA), a large protein on the surface of cells, is also needed for biofilm, formation evasion, and colonization. BapA (biofilm, association protein) and the csgD gene (curli, subunit gene) csgD is the master regulator of biofilm formation. It controls cell clustering by directly affecting how curli and fimbriae are made. It is similar to FimH. (15). The fact that bacteria live in harsh environments makes the biofilm important, and their ability to grow in, biofilms that stick to surfaces and are protected by an extra cell matrix, is key to their survival. The current results showed that all *S. typhi,* isolates were positive for the BapA (biofilm association protein) gene when they were, put through a standard PCR test. Biofilm-related, genes PCR was, used to find BapA and csgD. Strains that make biofilms were also studied to see what they were like. The results showed that *Salmonella enterica*, biofilm formation, is typical in isolates. This could be one reason it has spread so much in this country. Biofilm formation and biofilm-forming bacteria in salmonellosis infections lead to a steady rise in infections caused by microorganisms resistant to antibiotics.This study did agree with the study in BapA (10,21) and agree with the detection csgD gene (22) and agree with, (3). Disagree with (39) negative for the csgD gene because bacteria isolated from pigs and chicken. CsgD is involved in regulating several genes that change growth, characteristics like the formation of flagella, the structure of the cell surface, and some functions that help the cell respond to stress (40,41). Csg is the main transcriptional controller of biofilms. It controls how curli, cellulose, and other polymers are made in, Salmonella (42). The central biofilm formation bacteria belonged to the Enterobacteriaceae family and represented almost Gram-positive, isolates. The investigation into the formation of biofilm, In-vitro showed that all of the isolates had a high ability to form biofilms. The ability to form biofilms in the environment depended on the results of a PCR test that looked for the biofilm, association protein (BapA), and csgD genes. The major biofilm formation bacteria were belonged to Enterobacteriaceae family and represented, almost Gram positive, isolates. The investigation, into the formation of biofilm, *In-vitro* showed that all of the isolates had a high ability to form biofilms. The ability to form biofilms in the, environment depended on the results of a

PCR test that, looked for the biofilm, association protein, (BapA) and csgD gene.

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التنميط المظهري والجيني للسالمونيلا المعوية المنتجة للبايوفيلم المعزولة من الإسهال الحيواني والبشري

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الخلاصة

صممت الدراسة الحالية لتقدير تأثير ظروف الإجهاد المختلفة (درجة الحرارة ودرجة الحموضة) على تكوين الأغشية الحيوية للسالمونيلا المعوية من أصل 25 S. enterica بما في ذلك 12 عزلة من الحيوانات و13 عزلة من الإنسان. تم تقييم تكوين الأغشية الحيوية المظهرية والوراثية لعزلات *S. enterica* في مرق الصويا التجريبي (TSB) المحتضن عند 37 درجة مئوية و25 درجة مئوية و42 درجة مئوية باستخدام صفيحة ميكروترية من البوليسترين 96 بئر. كما تعرضت هذه العزلات لدرجات مختلفة من الأس الهيدروجيني (7، 5، 9 درجة حموضة) (أظهرت النتائج أن النسبة كانت 52٪ في الظروف القياسية (درجة الحرارة 37 درجة مئوية، ودرجة الحموضة 7) بينما في حالة أخرى لوحظت في نفس درجة الحرارة (37 درجة مئوية). لكن الأس الهيدروجيني يختلف (الأس الهيدروجيني 5، الرقم الهيدروجيني 9) لم ينتج عن *S. enterica* غشاء حيوي. أما بالنسبة لاستقرار الرقم الهيدروجيني في الرقم الهيدروجيني 7 مع تغير درجة الحرارة في 25 درجة مئوية، فإن إنتاج الأغشية الحيوية الرقيقة (44٪) بينما في 42 درجة مئوية (64٪) ثم تم استخدام معدلات الكشف عن الجينات بطريقه PCR ذات الصلة بالأغشية الحيوية لإيجاد جينات BapA و CsgDبنسبة 100٪، وبناءً على النتائج في هذه الدراسة قد اعطت معلومات مفيدة عن تكوين الأغشية الحيوية للسالمونيلا المعزولة من الحيوان والإنسان.

الكلمات المفتاحية: سالمونيلا، بايوفلم، جين،*CsgD* جين *BapA*.