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Assessment of Silver Nanoparticle as Anti-Salmonella Agent: Phenotypic and Genotypic Study

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ABSTRACT. Enteric serovars were both Salmonella enterica serovar typhi and Salmonella enterica serova typhimurium as effective human pathogens. They formed biofilm and multidrug-resistant. Bacterial biofilms have been demonstrated to be closely related to clinical infections and contribute to drug resistance. In the present study, we have analyzed S. typhimurium and S. typhi capability in forming a significant amount of biofilm, and the influence of AgNps , AgNps combined with Amoxicillin and Ciprofloxacin on biofilm development at sub inhibitory (Sub-MIC) concentration were inhibition of Salmonella biofilm and its impact on expressions of related genes (SirA, CsgD). 95 isolates of Salmonella sp. included, 63 of S. typhimurium and 32 of S. typhi isolates were used in the study. These isolates formed biofilm; it was demonstrated using a micro titer plate. Determination of its MIC(µg/ml) and at sub-MIC to AgNps , Amoxicillin ,Ciprofloxacin was demonstrated by microdilution methods. The coupled AgNps/Amoxicillin was calculated by chequer board method. The inhibited biofilm by sub MIC of antibacterial agent was observed in gene expression of SirA and CsgD by Q-Real time PCR. 95 isolates of S. typhimurium and S. typhi produced biofilms in phenotypic study including microplate titer methods, moreover the MIC level of AgNps was determined at (58.75,29.37) µg/ml, Mic of Amoxicillin (512,1024)µg/ml, Mic of Ciprofloxacin (0.5,0.25) µg/ml, and the FIC index values for AgNps, Amoxicillin combination were (0.56, 0.741) for S. typhimurium and S. typhi receptively. The genomic expression biofilm of Salmonella sp. changes were treatment at sub mic with AgNps ,Ciprofloxacin and Amoxicillin + AgNps, in comparison to samples control .These findings agreed with the recent findings that AgNps combined with Amoxicillin and Ciprofloxacin are the agent causing inhibited biofilm formation in S. typhi and S. typhimurium.

Key word. Salmonella, gene expression, AgNps, biofilm formation

INTRODUCTION

Both *Salmonella enterica serovar Typhi* and *Salmonella enterica serova Typhimurium* were enteric serovars as important human pathogens. The genus *Salmonella* incorporates Gram-negative, facultative anaerobic rod shaped bacilli that were members of the family *Enterobacteriaceae* [1].*Salmonella* enterica represents a major human and animal pathogen. It was responsible for two types of disease in humans due to the ingestion of contaminated food or water, gastroenteritis is caused mainly by *S. enterica serovar Typhimurium*, or enteric fever (typhoid fever), a severe systemic infection caused mainly by *S. enterica serovar Typhi* [2]. Owing to increasing of antibiotic resistance, researchers tend to find a successful alternative, of these; the nanotechnology. Silver nanoparticles (AgNPs) were highly toxic to Gram-negative and Gram-positive microorganisms, including multidrug resistant bacteria. Silver nanoparticles (AgNPs) had broad-spectrum bactericidal toxic effects against broad-spectrum pathogenic bacteria [3]. It was a non- toxic and safe antibacterial agent for the human body [4]. The combination of AgNps/Amoxicillin against various pathogenic bacteria inhibits the formation of biofilm that is associated with the resistance to antimicrobial agents and chronic bacterial infections [5]. The AgNPs generate hydroxyl radicals to enhance the bactericidal effect

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Chelation of AgNps is believed to prohibit DNA unwinding leading to bacterial cell damage, but a satisfactory synergistic mechanism is lacking [6].

MATERIAL AND METHODS

Samples and Patients

This study includes 213 samples that were collected from blood, stool and urine with clinical suspected suffering from typhoid fever and gastroenteritis from both sexes between the ages of (10 - 60) years old according to [7]. All samples were collected in the period from August 2018 to September 2019, who attended from Al-Ramadi Teaching Hospital Laboratory, Laboratory from Ramadi special private .All samples were collected under supervision of clinical consultant physician. The confirmed diagnosis was done by Vitek-2 compact system as table (1)

TABLE 1. Isolation and identification of *S. typhi* and *S. typhimurium*

Total samples: 239(100)%			
Total samples of positive culture of Salmonella isolates= 95(39.7)%			
Type samples	Total No. of Salmonella isolates &%	Total No. of S. typhi&%	Total No. of S. typhimurium&%
Stool	71	8	63
Blood	23	23	0
Urine	1	1	0
Total	95(100)%	32(33.7)%	63(66.3)%

Phenotypic and Genotypic studies of *S. typhimurium* and S. typhi isolates

Evaluation of Biofilm Formation by a Microtiter plate method according to [8] and determination Minimal inhibitory concentration (Mic) test and antimicrobial susceptibility *Salmonella typhi and S. typhimurium* identification and antimicrobial susceptibility testing of all drugs were performed again at AL-Ramadi teaching hospital for child and maternity laboratories by VITEK 2– compact (bioMérieux, France) .

This study used the microdilution broth technique to measured activity of an antimicrobial agent test (Ciprofloxacin, Amoxicillin, and AgNps) in vitro according to guidelines CLSI [9]. While, genotypic study includes PCR assay performed to identify some virulence genes (invA, fimA, stn, and hilA) and detection of some biofilm formation genes (csgD and sirA)) in the Salmonella isolated. Quantitative Real time PCR was used to detect SirA and CsgD genes of both S. typhimurium and S.typhi, respectively with specific primer according to recommendations of company instructions.

Silver Nanoparticles Synthesis

Synthesis of AgNps was achieved according to [11].

Estimation Synergistic Effects between Amoxicillin and AgNPs against *Salmonella* Isolates

The combination AgNps-Amoxicillin used to assess gene expression by chequer board methods. The sub MIC (0.5 MIC) assessed of gene expression of biofilm formation in Salmonella spp. [12]. The Salmonella typhi and S. typhimurium are treated at Sub- MIC of (Ciprofloxacin, AgNps and combination between AgNps/Amoxicillin) were

called positive samples, while the samples without treatment of Sub MIC described above as called control samples. All samples were estimation expression genes of CsgD, SiRA by Quantitative - PCR.

RESULT AND DISCUSSION

In the present study, after collection of 95 of Salmonella SPP. included, 63 of S. typhimurium and 32 of S. typhi isolates and identification by the Vitek-2 compact system techniques, their biofilm-producing ability was evaluated using microtiter plates were detected highly positive results compared with negative result in table (2) and Fig. 1. These results were showed *Salmonella* isolates were able to form biofilm on plastic surfaces with non-significant p value >0.01.This results are in agreement with [13].

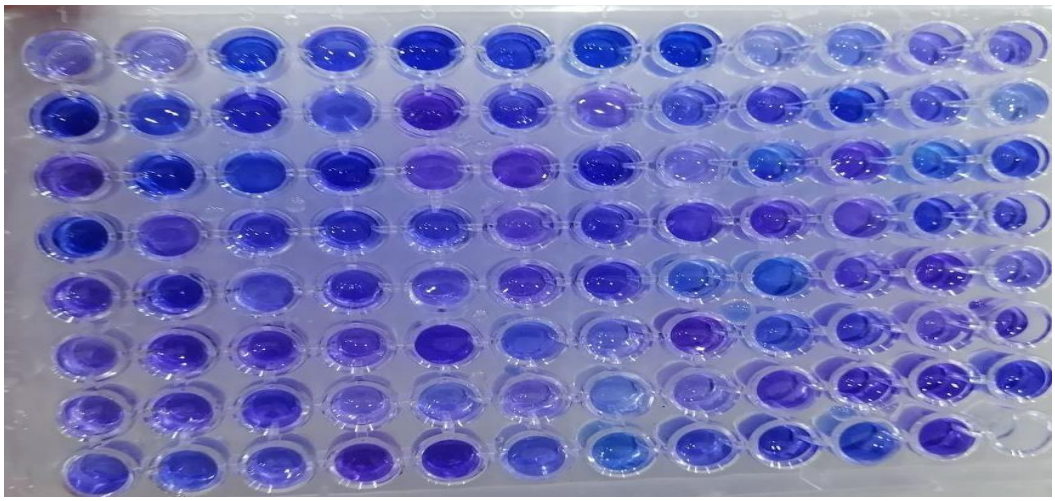

FIGURE 1. Ability of *S.typhi* to biofilm formation by microplate titer methods

TABLE 2. Biofilm formation of *S. typhimurium* and *S. typhi* by microplate titer assay

Bacteria	No. of bacteria	NO.&% Positive biofilm	NO.&% Negative biofilm	NO.&% of Weak biofilm	NO. &% of Moderate biofilm	No. &% of Strong biofilm
S. typhi	32	27(84.37)	5(15.63)	3(11.15)	14(51.85)	10(37.0)
S. typhimrium	63	55(87.3)	8(12.7)	8 (14.5)	26(47.3)	21(38.2)
Total	95	82(86.3)	13(13.7)	11(13.4)	40(48.8)	31(37.8)

Antibiogram Testing (MIC) of S. typhi by VITEK-2 Compact Approaches

The results of antibiogram profile of 32 *S.typhi* isolates were achieved in VITEK-2 Compact, indicated that Salmonella isolates were varied in their susceptibility to the antibiotics. The S. typhi isolates were highly sensitive to (87.5, 78.1, 68.7, 65.6, 62.6) % imipenem, meropenem, Ciprofloxacin, (aztreonam and cefepime) and Amikacin receptively, shown in table (3). About of 63 *S. typhimurium* isolates were highly sensitive (85.7, 81.0, 69.8, 66.7, 65.1, 63.5, and 60.3) % to meropenem, imipenem, cefixime, piperacillin, TM-SULF, Azet and Amikacin, respectively. All

susceptibility testing were shown in table (2). There are significant differences found in antibiotic sensitivity with among resistant, sensitive and intermediate values (P-value < 0.005). The high sensitivity of imipenim and meropenime is due to these antibiotics not being common in the community and are very expensive and not used indiscriminately because not many can afford those [14]. Almost antibiotic resistant, this may be as a result of indiscriminate use of these antibiotics and the over use of these drugs in human medicine over a long period. Self-medications are another factor that may account for this high level of resistance to these antibiotics as well the development of the MDR organisms [15]. Most *Salmonella* enterica strains were susceptible to Ciprofloxacin, amikacin and levofloxacin among patients. These results are in agreement with [16].

TABLE 3 Antibiogram Testing (MIC) of S. typhi and S. typhimurium by VITEK-2 Compact approaches

	Total number of *Salmonella typhi*=32			Total number of *Salmonella typhimurium*=63		
Antibiotic	No. isolates & MIC % in sensitive antibiotic	No. isolates &MIC value% in intermediate antibiotic	No. isolates &MIC value % in resistance antibiotic	No. isolates & MIC value % in sensitive antibiotic	No. isolates& MIC value in intermediate antibiotic %	No .isolates & MIC value % in resistance antibiotic
AK	20 (62.6)	5 (15.6)	7 (21.8)	38 (60.3)	19(30.2)	6(9.5)
AMP	10 (31.3)	5 (15.6)	17 (53.1)	21 (33.3)	10(15.9)	32(50.8)
AZET	21 (65.6)	8 (25.0)	3 (9.4)	40 (63.5)	9(14.3)	14(22.2)
CFP	21 (65.6)	6 (18.8)	5 (15.6)	35 (55.6)	13(20.6)	15(23.8)
CFX	13 (40.6)	14 (43.8)	5 (15.6)	44 (69.8)	12(19.1)	7(11.1)
CH	17 (53.1)	5 (15.6)	10 (31.3)	31 (49.2)	22(34.9)	10(15.9)
CIP	22 (68.7)	7 (21.9)	3 (9.4)	32 (50.8)	18(28.6)	13(20.6)
CRO	11 (34.3)	15 (46.9)	6 (18.8)	35 (55.6)	15(23.8)	13(20.6)
Gent	19 (59.4)	4 (12.5)	9 (28.1)	33 (52.4)	20(31. 7)	10 (15.9)
IMP	28 (87.5)	3 (9.4)	1 (3.1)	51 (81.0)	7(11.1)	5(7.9)
MRP	25 (78.1)	4 (12.5)	3 (9.4)	54 (85.7)	6 (9.5)	3(4.8)
PIP	18 (56.2)	10 (31.3)	4 (12.5)	42 (66.7)	11(17.4)	10(15.9)
Tig	15 (46.9)	9 (28.1)	8 (25.0)	29 (46.0)	16(25.4)	18(28.6)
TM-SULF	16 (50.1)	9 (28.1)	7 (21.9)	41 (65.1)	10(15.9)	12(19.0)
TRIM	17 (53.1)	7 (21.9)	8 (25.0)	31 (49.2)	15(23.8)	17(27.0)

Determination of Antimicrobial Susceptibility Test (MIC) by Micro Broth Dilution

In the present study that measured anti-bacterial effect of Ciprofloxacin, Amoxicillin and AgNps against *S. typhi* and *S. typhimurium* were calculated by VITEK-2 Compact approaches and micro dilution method according [9]. The MIC results showed in table (3) and Fig. 2 (a, b, c, d, e, and f).The MIC could be used in determination gene expression of *Salmonella* isolates, by using it in a Sub MIC. These results are in agreement with [17].

Anti-Bacterial Activity of Silver Nanoparticles Combined with Amoxicillin

The synergistic effects of AgNps were achieved in the combination of Amoxicillin with AgNPs against *S. typhi* and *S. typhimurium* by using chequer board assay and the effects evaluated by determination of the FICI. The FIC index values for AgNps, Amoxicillin combination were 0.74 and 0.56 for *S. typhi* and *S.typhimurium*, Respectively. The additive effects were excessed in antibacterial activities of Amoxicillin, in combination with the biosyhthesized

of AgNPs against *Salmonella spp.* [18]. The combination was used to estimate the gene expression of bacteria.

Genotypic Identification of Virulence Genes

The present study of S. *typhi* and *S.typhimurium* isolates were detected (*invA*, *hilA*, *SirA*,*FimA*, *Stn* and *CsgD*) by PCR techniques. All genes had positive results for all Salmonella isolates were showed in Fig. 3 (a, b, c, d, e, and f). This result agrees with the findings of many investigators [19, 20].

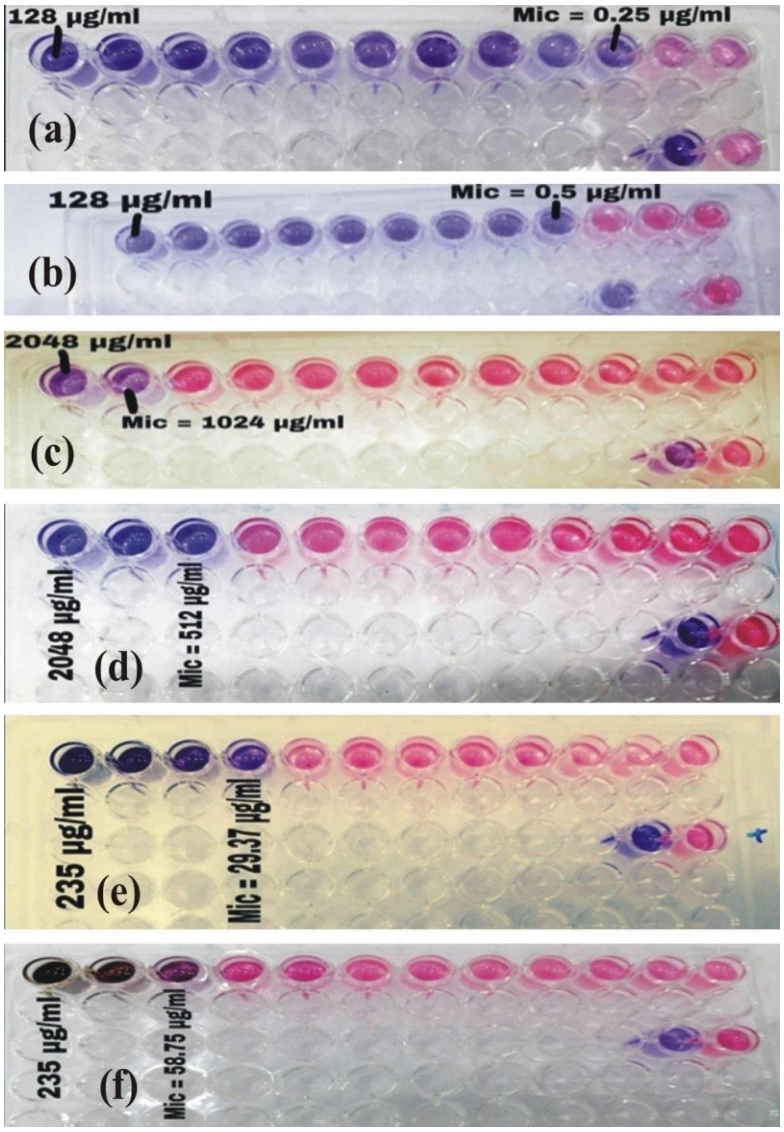

FIGURE 2, (a) Mic of Ciprofloxacin for S. typhi =0.25 µg/mL, (b) Mic of Ciprofloxacin for S. typhimurium =0.5 µg/mL, (c) Mic of Amoxicillin of S.typhi =1024 µg/mL, (d) Mic of Amoxicillin of S.typhimurium = 512µg/mL, (e) Mic of AgNps in S. typhi, with Mic=29.37 µg/ ml, and (f) Mic of AgNps in *S. typhimurium*, with Mic=58.75 µg/ ml

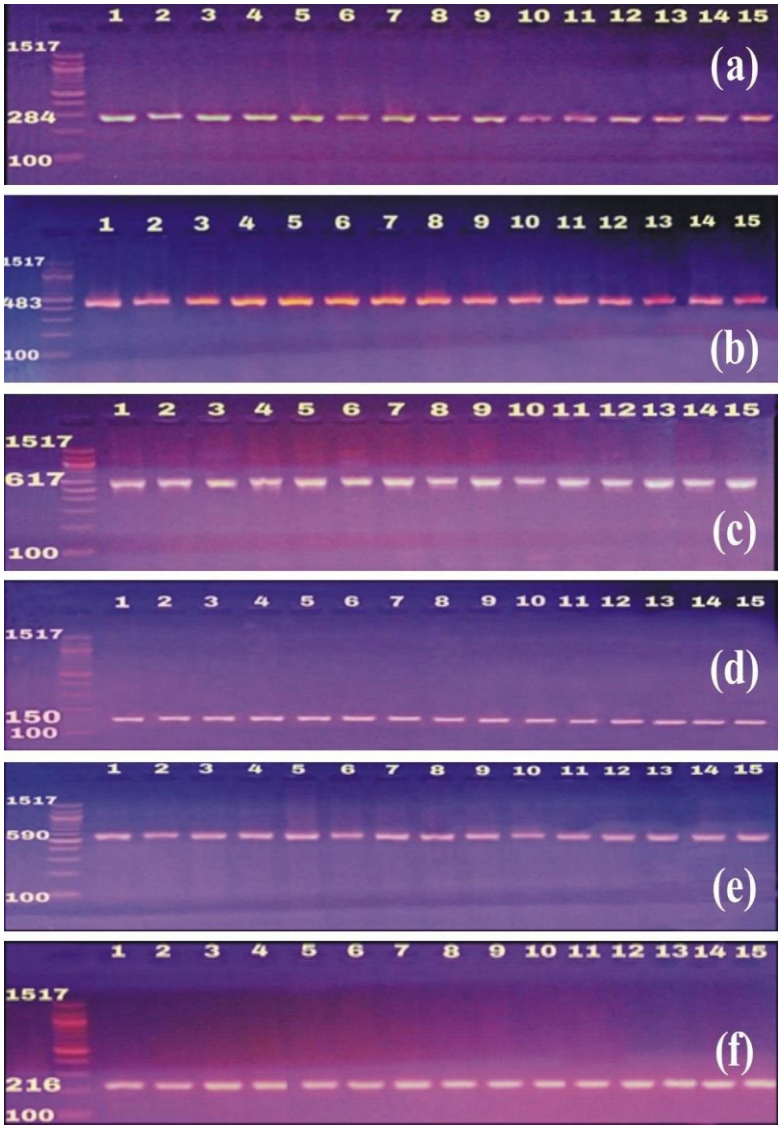

FIGURE 3. Amplification products of (a) InvA gene of S. typhi by PCR with product 284bp, DNA Ladder(100bp), agarose conc.(1.5%) and Ethedium bromid stain ; electric current at 60 volts for 85 mint, (b) fimA gene of S. typhi by PCR with product 483bp, DNA Ladder(100bp), agarose conc.(1.5%) and Ethedium bromid stain ; electric current at 60 volts for 85 mint, (c) stn gene of S. typhi byPCR with product 617bp, DNA Ladder(100bp), agarose conc.(1.5%) and Ethedium bromid stain ; electric current at 60 volts for 85 mint, (d) hilA gene of S.typhi byPCR with product 150bp,DNA Ladder(100bp), agarose conc.(1.5%) and Ethedium bromid stain ; electric current at 60 volts for 85 mint, (e) CsgD gene of S.typhi byPCR with product 590bp, DNA Ladder(100bp), agarose conc.(1.5%) and Ethedium bromid stain ; electric current at 60 volts for 85 mint, and (f) SirA gene of S.typhi byPCR with product 216bp, DNA Ladder(100bp), agarose conc.(1.5%) and Ethedium bromid stain ; electric current at 60 volts for 85 mint

Assessment Gene Expression of CsgD Genes of *S. typhi* and *S. typhimurium*

S. typhi gene expression level of *CsgD* gene with mixed of AgNps/Amoxicillin treatment at Sub Mic concentration was (0.514)) in contrast with AgNps and Ciprofloxacin treatments were (0.312 and 0.216) respectively, all results above were compared with the gene expression of control samples (without treatment) at folding changes (1.0) in

table (4). However, gene expression of CsgD gene in *S. typhimurium with* AgNps/Amoxicillin treatment at Sub Mic concentration was (0.098) in contrast with AgNps and Ciprofloxacin treatments as (0.507 and 0.021) respectively, all result above were compared with the gene expression of control samples at (1.0)in table(5).

TABLE 4. *S. typhi* Gene expression values of CsgD genes

Type of bacteria	Treatment	csgD	*Rpo*	DCt	DDCt	FOLD CHANGE
S. typhi	Ag-after	14.17	14.75	0.58	1.68	0.312
	Cip-after	19.33	20.44	1.11	2.21	0.216
	Mix	12.86	12.72	-0.14	0.96	0.514
	Control	15.85	14.75	-1.1	0	1.000

TABLE 5. *Salmonella typhimurium* Gene expression values of CsgD genes

Type of bacteria	Treatment	csgD	*Rpo*	DCt	DDCt	FOLD CHANGE
S. typhimrium	Ag-after	15.84	16.21	0.37	0.98	0.507
	Cip-after	14.54	19.52	4.98	5.59	0.021
	Mix	14.65	17.39	2.74	3.35	0.098
	Control	15.69	15.08	-0.61	0	1.0

According to *S. typhi*, the sub-MICs of CIP was inhibited biofilm formation through inhibiting the expression of *CsgD* genes resulting in decreased extracellular polysaccharide and curli produced. This result is in agreement with [21]. The sub-MIC of AgNPs, combined AgNps/Amoxicillin and Ciprofloxacin significantly inhibited the growth rate of biofilm formation of *S.typhi* due to decreased gene expression of *S.typhi* related to motility and biofilm formation [22]. The study results of *S. typhimurium* gene expression at Sub-Mic of combined AgNps/Amoxicillin showed significant antibacterial activity .It was decreased gene expression of *S.typhimurium*. Due to the cell membrane consisting of phospholipids and glycoprotein, which are all hydrophobic groups. Therefore, antimicrobial groups facilitate the transport of Amoxicillin to the cell surface [23]. The results demonstrates that AgNPs at sub-MIC concentrations lead to partial killing and decreased gene expression of *S,typhimurium* and inhibition of biofilm formation and bacterial growth. The results are in agreement with findings of [24]. The sub-MICs of CIP was inhibited biofilm formation by inhibiting the expression of *CsgD* genes resulting preventing DNA synthesis and subsequently decreased biofilm formation release [25]. These results are in agreement with [26].

Assessment Gene Expression of *SirA* gene *of S. typhi* and *S. typhimurium*

The present study, showed significant decrease in *SirA* gene expression level (0.204) of *S. typhi* in combination the AgNps/Amoxicillin treatment at Sub-Mic concentration ,in contrast with AgNps and Ciprofloxacin treatments at(0.376 and 0.191) respectively, all result above were compared with the gene expression of control samples without treatment at (1.0) in table (6).

TABLE 6. Gene expression values of *sirA* genes of *S.typhi*

Type of bacteria	Treatment	csgD	*Rpo*	DCt	DDCt	FOLD CHANGE
S. typhi	Ag-after	14.29	14.57	0.28	1.41	0.376
	Cip-after	19.18	20.44	1.26	2.39	0.191
	Mix	11.56	12.72	1.16	2.29	0.204
	Control	15.88	14.75	-1.13	0	1.000

The AgNps chelates prevented the DNA from unwinding, which result in damage of cells. Therefore, the sub inhibitory of coupled AgNps/Amoxicillin inhibited of gene expression. Ciprofloxacin affected on the biofilm formation and may be attribute to inhibit the DNA replication by inhibiting bacterial DNA topoisomerase and DNA-gyrase, lead to inhibit DNA synthesis [27]. The coupled AgNps/Amoxicillin caused biofilm inhibition of *S. typhi*, but its effect compared with AgNps was less than the latter. There was an opinion that AgNPs had the same mechanism of the antimicrobial agents, which include; inhibition of cell wall synthesis, nucleic acid, protein synthesis, and metabolic pathway [28]. These results are in agreement finding with [29]. In the same study about *S. typhimurium isolate* showed a strongly significant decrease in *SirA* gene expression in *S. typhimurium.* This study included the Sub Mic coupling AgNps/Amoxicillin treatment was an impact of gene expression level at (0.0000048) in contrast with Sub Mic of AgNps and Ciprofloxacin treatments as (0.883 and 0.018) respectively, all results above were compared with the gene expression of control samples at (1.0) in table (7).

TABLE 7. Gene expression values of *sirA* genes of *S.typhimurium*

Type of bacteria	Treatment	csgD	*Rpo*	DCt	DDCt	FOLD CHANGE
S. typhimurium	Ag-after	16.21	16.37	-0.16	0.18	0.883
	Cip-after	19.96	14.54	5.42	5.76	0.018
	Mix	14.65	17.34	17.34	17.68	0.0000048
	Control	15.42	15.08	-0.34	0	1.0

Results of sub -Mic concentrations showed; the gene expression inhibition of *SirA* and not form biofilm. Due to sirA was not regulated of motility and virulence state [30] and the SirA gene expression was used to regulate motility fimbrial gene (FlhD/C) and virulence through determining that hilA, these genes were not mainly regulated by the BarA/SirA pathway, and a frame-shift causing did not occur the gene expression of sirA[31], which is agreed with [32].

CONCLUSION

The study above clearly shows, the anti-biofilm agent of *S. typhimurium* included AgNps, Ciprofloxacin, and (Amoxicillin + AgNps) at sub-MIC level efficiently induces biofilm formation and promotes changes in morphology of the cell.

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