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Reconfiguring the quorum regulator SdiA In EHEC O157:H7 to curtail its invasion of Hep-2 cells via Qq SdiA^{m}

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ABSTRACT

Bacteria accomplish various tasks necessary for their survival through Quorum Sensing (QS), a Process that ensures synchronization of their activities at the genetic level, which brings about favourable phenotypic changes. Highly pathogenic organisms, such as Enterohaemmorrhagic *E.coli* (EHEC O157:H7), use QS as an important mechanism for establishing virulence and biofilm formation in host cells. These infections lead to haemorrhagic colitis, which could progress to a clinically critical condition termed Haemolytic Uremic Syndrome (HUS). Earlier investigation of our research group has implicated that compounds derived from *Melia dubia*, shows SdiA selective biofilm inhibition of Uropathogenic *E.coli* (UPEC). One such compound, QQ SdiAM, was tested as an SdiA antagonist of EHEC O157:H7 strains. QQ SdiAM was found to be effective at low concentration (MBEC of 1 µg/ml), and the results of biochemical assays performed showed that it has potent anti biofilm activity. Autoaggregation and cell surface Hydrophobicity reduced upon treatment, as did acid resistance. The % hemolytic activity also decreased in sdiA+ strain upon QQ SdiAM treatment. Swarming motility showed an increase upon treatment. Expression of FtsZ cell division protein decreased by 51% in sdiA+ strain. In all these tests, the compound showed little to no effect on the "sdiA strains. CLSM analysis of strains adhered to HEp-2 cells showed significant decrease in biofilm parameters such as thickness and biomass, in sdiA+ strain. The compound also had high % cell viability (92%) at its MBEC value. These results suggest not only that QQ SdiAM is a potent antagonist of SdiA, but it is an anti-biofilm compound as well.

KEYWORDS

Quorum Sensing;
EHEC O157:H7;
SdiA;
QQ SdiA^{M}.

INTRODUCTION

Bacteria have evolved over the course of time in order to optimize their survival and proliferation under extreme environmental conditions[1], perfecting their existence as complex communal units called biofilms, which are polymer matrices embedded with individual cells[2]. Most pathogenic bacteria communicate with each other through extracellular signalling molecules in order to perform coordinated functions, called quorum sensing (QS)[3]. These interactions lead to enhanced virulence, biofilm formation, antibiotic resistance, acid resistance and heightened cell survival under harsh conditions present in the host[4].

Enterohaemmorrhagic *E.coli* (EHEC O157:H7), a gram negative bacterium that produces a Shiga-like toxin called verotoxin, is a deadly pathogen, with infectious doses as low as 2-100 bacteria[5]. Its symptoms include bloody diarrhoea, which progresses to haemorrhagic colitis and Hemolytic Uremic Syndrome (HUS), which can be fatal to young children and the elderly[6]. HUS, which is characterized by vomiting and diarrhoea, may lead to kidney failure and haemolytic anaemia. The major virulence of EHEC O157:H7 is conferred by a 35.5 kbp island in the chromosome, called Locus of Enterocyte Effacement (LEE) Pathogenecity Island (PAI)[7]. The proteins produced by this island form the core of transport of toxins into host cells through the type 3 secretion system. EHEC O157:H7 forms intimate attachment with the host cells through adhesion proteins from LEE PAI, resulting in the formation of Attaching\Effacing (A\E) lesions along the lumen of the intestine. EHEC possesses a 90 kbp plasmid pO157 that encodes for verotoxins and haemolysins, also contributing to its virulence.

Based on the signalling molecules, QS systems in bacteria can be classified into 3 types, the Autoinducer-1 (AI1) AHL pathway, the Autoinducer-2 (AI2) pathway and the Autoinducer-3(AI-3) pathway[8]. SdiA (suppressor of cell division inhibition), the QS protein of E.coli, is a LuxR homolog involved in intraspecific communication through indole by AI-2 pathway, and interspecific communication through AHLs[9], by AI-1 pathway. It has been shown that certain genes of *E.coli* and EHEC O157:H7 responsible for virulence and biofilm formation are under the control of SdiA. Studies suggest that SdiA represses the expression of virulence factors during the stationary phase[9], enhances multidrug resistance[10], increases acid tolerance and also increases the biofilm formation.

Other characteristics of EHEC O157:H7 strains which influence its attachment and biofilm formation that may also be under the control of SdiA include autoaggregation, cell surface hydrophobicity, motility and acid resistance. Accordingly, the genes encoding for some of these characteristics, such as *eps, fim, wza* and *caf*, seem to be under SdiA control. We hypothesize that SdiA, acting as a global controller, regulates the biofilm formation and establishment, and that it has some effect upon virulence as well. Hence, a suitable SdiA antagonist would nullify its effect, and reduce the pathogenecity of EHEC O157:H7 strains. Our goal is to validate the role of QQ $SdiA^M$, a compound derived and purified from *Melia dubia*, as an EHEC SdiA antagonist, to determine its MBEC concentration and evaluate its anti-biofilm potential through biochemical and *in vitro* HEp-2 cell adherence assays.

MATERIALS AND METHODS

Bacterial strains and medium conditions

The strains used are shown in TABLE 1, which includes EHEC O157:H7 *sdiA*$^+$ wild type strain and the mutant *"sdiA* strain, gifted by Dr.Thomas K.Wood of Pennsylvania State University. The cells were revived from slants and successive experiments were performed using Luria Bertani (LB) medium.

TABLE 1 : Strains used in this study

Strain	Description	Reference
$sdiA^+$	Wild type strain of EHEC O157:H7 containing sdiA	[12]
ΔsdiA	Mutant strain lacking sdiA	[12]

Studies on growth

The strains were cultured in LB medium and the Optical Density (OD) was measured at 600 nm at regular time intervals for a period of 24 hours. Simultaneously, to measure the CFU/ml, colonies were counted in LB agar at the same time intervals.

Biofilm assay

The biofilm assay was adapted from the method

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followed by Silagyi *et al*[11]. Briefly, overnight cultures were diluted in LB broth and aliquoted in 96 well microtitre plates, with or without addition of QQ SdiAM. After 24 hours, the medium was discarded and wells were washed twice with sterile PBS, and adherent cells were stained with 0.1% crystal violet in 90% ethanol. After incubation at RT for 30 minutes, OD was measured at 590 nm.

The results were quantified using specific biofilm forming index[12] (SBF), as $SBF = (AB - CW)/G$, where AB=test culture, CW= uninoculated medium, G= inoculated medium.

Minimum biofilm eradication concentration (MBEC) assay:

The minimum biofilm eradication concentration, at which bacteria fail to regrow after addition of antimicrobial compound, was determined using tube assay and microtitre plate assay[13]. Overnight cultures of both strains were diluted in 1/100 ratio and incubated for 24 hours at 30ÚC. The medium was aspirated, and wells were washed with distilled water twice. The drug QQ $SdiA^M$ was added at different concentrations ranging from 5 ng/ml to 1.2 µg/ml. After incubation with drug for 24 hours, the wells were washed thoroughly with sterile saline and 1% peptone was added to the wells. OD was taken at 655 nm after 24 hours. The same assay was also performed in test tubes. CFU/ml was also measured in LB agar plates after 24 hour incubation with peptone.

Autoaggregation assay

Autoaggregation assay was performed according to Del Re *et al.*[14]. 10 ml of 18th hour (late exponential phase) cultures treated with/without MBEC of QQ $SdiA^M$ were placed on ice and 100µl of samples were withdrawn 1 cm below the culture level, at regular time intervals, and transferred aseptically to new tubes containing 1 ml of sterile saline containing 9% NaCl. The OD was then recorded at 600 nm.

The % autoaggregation was calculated as per the formula $\%AA = \left[\frac{OD0 - OD600}{OD0}\right] \times 100$

Cell surface hydrophobicity:

The cell surface Hydrophobicity was adapted from Gogra *et al.*[15] To check the % Hydrophobicity, the overnight treated and untreated cultures were centrifuged and resuspended in sterile PBS. The OD at 660 nm was adjusted to 0.4, and 2.5 ml of bacterial culture was mixed vigorously with 1 ml of p-xylene for 2 minutes. Then, it was allowed to stand at RT for 20 minutes, whereby it separates into two phases. The aqueous phase was removed and its OD at 660 nm was determined spectrophotometrically.

Quantitatively, the extent of hydrophocity is calculated as $\%HI = \left[\frac{A660control - A660test}{A660\ control}\right] \times 100$

Hemolysis assay

EHEC O157:H7 strains were inoculated in LB media overnight, supplemented with MBEC of QQ SdiAM. 50 µl of 2 fold dilutions of culture in saline containing 5 mM of Calcium Chloride. Equal volume of 5% human RBC suspended in PBS was added, and centrifuged at 750 x g for 15 minutes. The supernatant was collected and its OD at 570 nm was measured.

% Lysis was calculated using the formula

$$\%Lysis = \left[\frac{ODsample - ODbackground}{ODtotal - ODbackground}\right] \times 100.$$

Total hemolysis is produced by distilled water, and background hemolysis is the amount of lysis produced by the Calcium chloride alone.

Acid sensitivity assay

Exponential phase treated and untreated cultures of EHEC O157:H7 strains containing approximately 10^{10} CFU/ml were centrifuged and washed with equal volume of LB or saline[16]. Resuspend the cultures in same volume of sterile LB broth with pH adjusted to 2.5 using 1M HCl. These are designated as undiluted cultures. From the centrifuged samples, dilution was also done (100-fold) in acidified LB medium; these are designated as diluted cultures. These cultures are incubated at 37ÚC overnight with shaking in 100 ml conical flasks. Samples were periodically withdrawn and diluted appropriately in 0.85% saline, and colonies were counted on LB agar plates. The extent of colonies growing in LB plates after 24 hours will give an indication of acid sensitivity of the treated cultures.

Swarming motility

The swarming motility of EHEC O157:H7 strains were tested on LB agar plates containing 0.4% agar.

Overnight LB cultures of the untreated and treated strains (with MBEC of QQ SdiA^M), were washed twice with sterile PBS, and 50 µl of sample was pipetted into the plates and incubated at 37ÚC for 24 hours. The bacterial motility was measured as the distal extent of movement by the bacteria, in cm.

SDS Page electrophoresis:

To determine the fold expression of FtsZ protein in the treated and untreated cultures, 100 ml of overnight cultures were centrifuged and the pellets were collected. These were dissolved in 3 ml of cell lysis buffer, and incubated for 15 minutes. This mixture was again centrifuged, and the supernatant was collected. The individual supernatants were then subjected to ammonium sulphate precipitation at a20% saturation, and the pellets collected, containing inactive FtsZ proteins were discarded[17]. The supernatants obtained as a result of ammonium sulphate precipitation were further subjected to precipitation, by increasing the saturation % of ammonium sulphate to 25%. The resultant 25% pellets were suspended in appropriate volume of sterile PBS and stored at -20ÚC till further use. These pellets, containing active FtsZ proteins, were run in SDS PAGE, containing 10% separating gel.

In vitro cell line studies

HEp-2 cell adherence assay

The HEp-2 cells were purchased from NCCS, Pune. The cells were grown in DMEM medium until confluency was obtained. The cells were maintained in DMEM containing antibiotics till use[18]. When cells reached confluent stage, they were added to 6 well plates containing sterile cover slips and incubated in CO_2 incubator for 24 hours. After incubation, FITC labelled treated bacterial cultures were added to the cover slips with and without drug. The wells were again incubated in CO2 incubator for 3 hours, and then the cover slips were aseptically removed and used for CLSM analysis. COMSTAT software with MATLAB was used to analyse biofilms.

Cell cytotoxicity assay

The cytotoxicity of the drug towards HEp-2 cells was treated using two concentrations, MBEC concentration, and high dose (100 times MBEC). The value of high dose was set arbitrarily. The MTT proliferation assay was followed[19]. Briefly, the sub confluent HEP-2 cells were added in microtitre plate wells, along with DMEM medium alone. After adherence was established in the plates (24 hour incubation in CO2 incubator), the drug was added at the fixed concentrations to the wells, followed by addition of MTT and incubation for 3 hours in CO2incubator. Following the incubation period, 200 µl of isopropanol was added to the wells and OD was taken at 590 nm.

RESULTS

Figure 1 shows the growth pattern of EHEC O157:H7 strains along a period of 24 hours, differentiated into different growth phases for each of the strains. Quantitatively, the number of colonies of both strains were determined, and the 24 hour CFU/ml of both strains were of the order of 3-4 × 10^{10} colonies, as represented in TABLE 2. The results of biofilm formation (Figure 2) without addition of drug shows that sdiA+ strain produced moderate biofilms compared to mutant strains producing weak biofilm, as quantified by the SBF values.

The MBEC of QQ SdiAM was determined, using

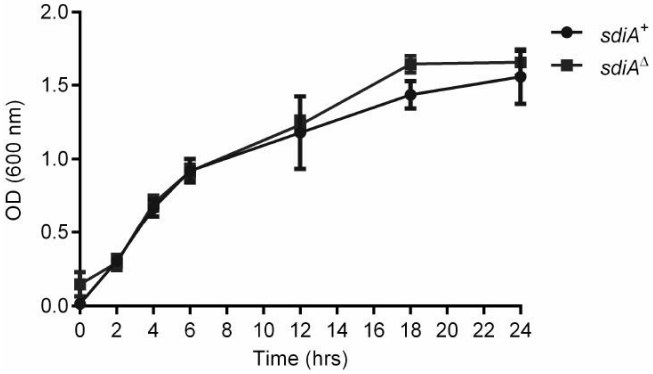

Figure 1 : Time dependent growth pattern of EHEC O157:H7 strains in Luria broth

TABLE 2 : Viable counts (Colony Forming Units (CFU/ml)) of EHEC O157:H7 strains

Growth Phases	CFU/ml	
	sdiA$^+$	ΔsdiA
Early Exponential	6.6 x 10^5	5.0 x 10^5
Mid Exponential	5.8 x 10^7	5.5 x 10^7
Late Exponential	3.8 x 10^8	4.3 x 10^8
Stationary	3.6 x 10^{10}	3.9 x 10^{10}

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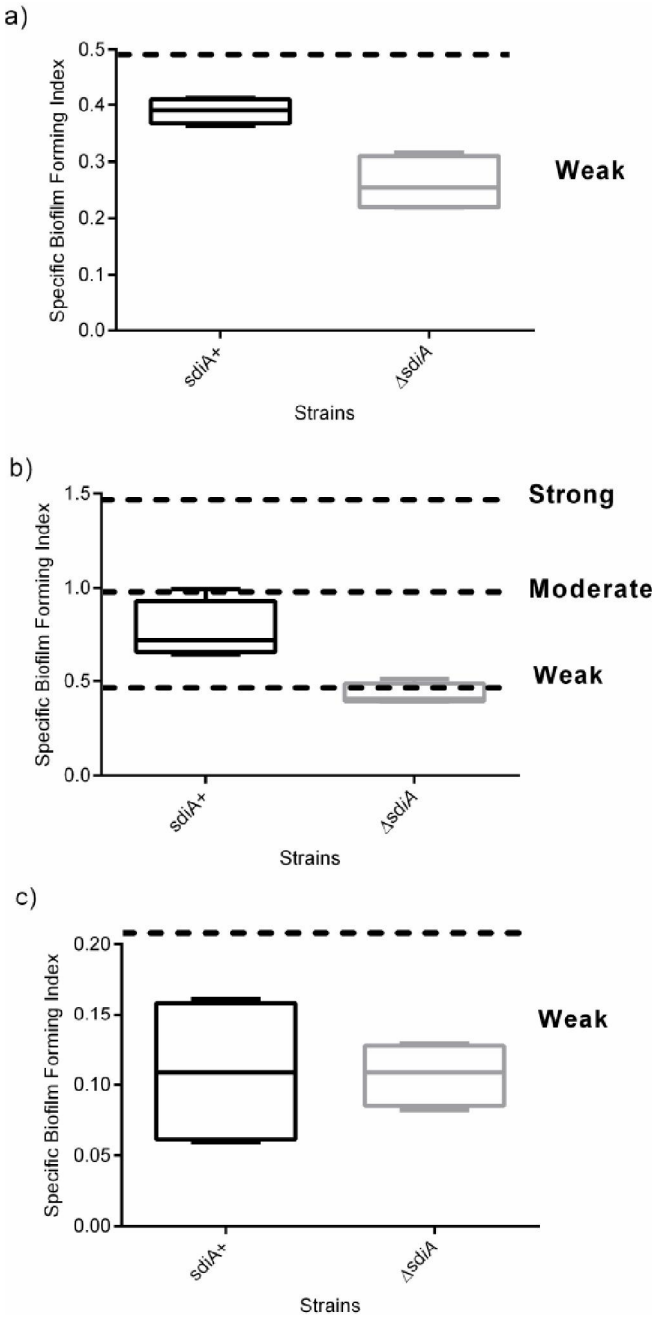

Figure 2 : Mean Specific Biofilm Forming Index of EHEC O157:H7 strains at various growth phases (A-Mid exponential; B-Late exponential; C-Stationary)

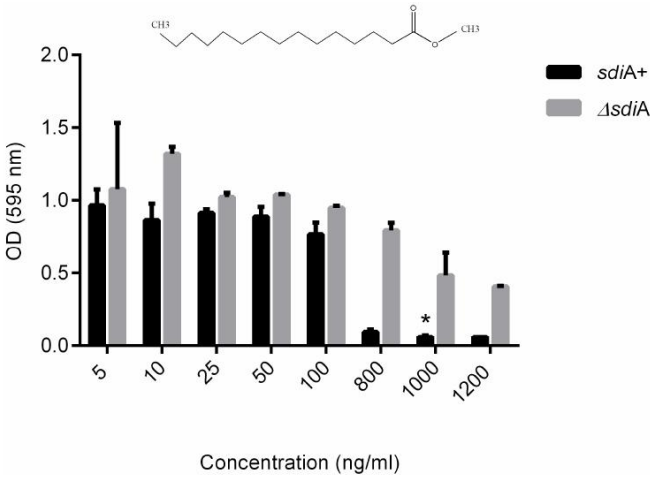

Figure 3 : Effect of SdiA antagonist QQ SdiAM treatment on determining Minimum Biofilm Eradication Concentration (MBEC) on EHEC O157:H7 strains in the late exponential phase

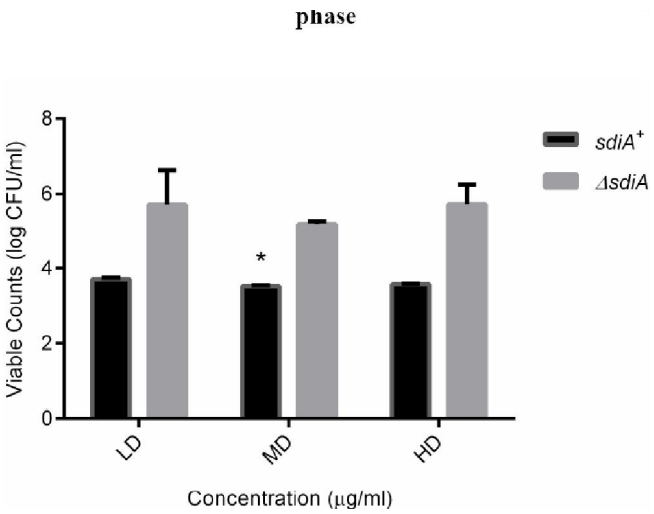

Figure 4 : Effect of QQ SdiAM (Low Dose (LD-0.8 ìg/ml); Medium Dose (MD-1 ìg/ml); High Dose (HD-1.2ìg/ml)) on cell viability (log CFU/ml) of EHEC O157:H7 strains

different concentrations of drug ranging from5 ng/ml to1.2 µg/ml. The results indicate that the biofilm was eradicated lowest when QQ SdiA^{M} concentration was 1 µg/ml, as shown in Figure 3 and Figure 4. Using the MBEC value, biofilm formation assay was repeated with addition of QQSdiA^{M,} and the result showed that a decrease in biofilm formation was observed only in the case of sdiA+ strain, with negligible effect on the mutant strain (Figure 5).

Autoaggregation and cell surface hydrophobicity, two phenotypes related to biofilm formation were tested using MBEC of QQ SdiA^{M}. Figure 6 and Figure 8 show that a decrease was observed in the sdiA+ strain with addition of drug, whereas no significant changes were observed in the mutant strain on addition of drug. A graph plotted between the biofilm readings and autoaggregation % showed a positive correlation, as seen in Figure 7. Hemolysis assay was quantitatively performed with the bacterial cells treated with QQ SdiA^{M}. The drug treated sdiA+ strains showed a marked decrease in % hemolysis (Figure 9). it was found that

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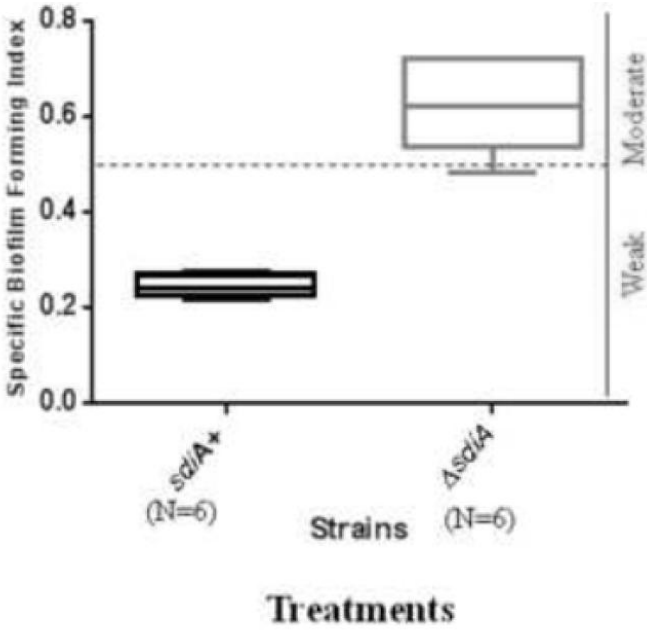

Figure 5 : Effect of QQ SdiAM (1 ìg/ml) on Mean Specific Biofilm Forming Index of EHEC O157:H7 strains

addition of QQ SdiA^M to sdiA+ cells increased its acid sensitivity, the sdiA+ control showed greater acid resistance, wheareas the mutant responses were more or less identical, as shown in Figure 10. Figure 11 shows an increase in swarming motility zone for drug treated sdiA+ strain, compared to mutant strain lacking SdiA. The effects of all the biochemical assays of treated and untreated sdiA+ strain are plotted in Figure 12.

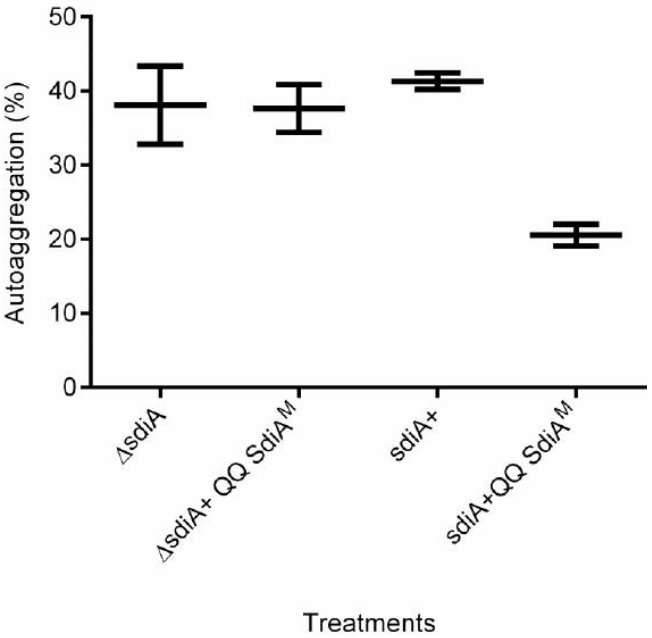

Figure 6 : The effect of QQ SdiAM (1 ìg/ml) on the autoaggregation ability of EHEC O157:H7 strains

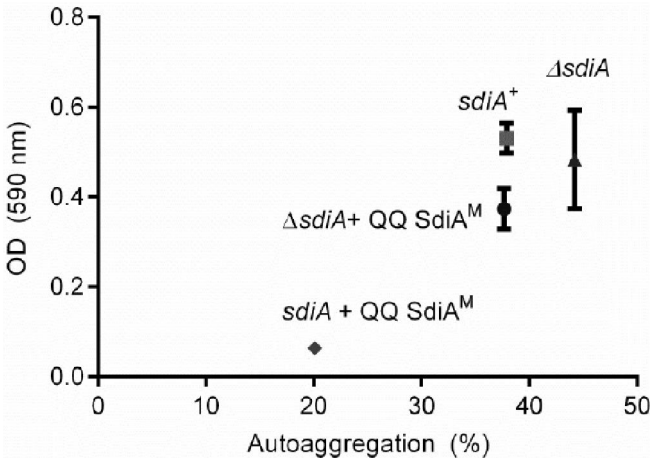

Figure 7 : Scatter Plot of 2 variables: autoaggregation % vs. biofilm formation. The diamonds are ordered pairs that represent EHEC O157:H7 strains

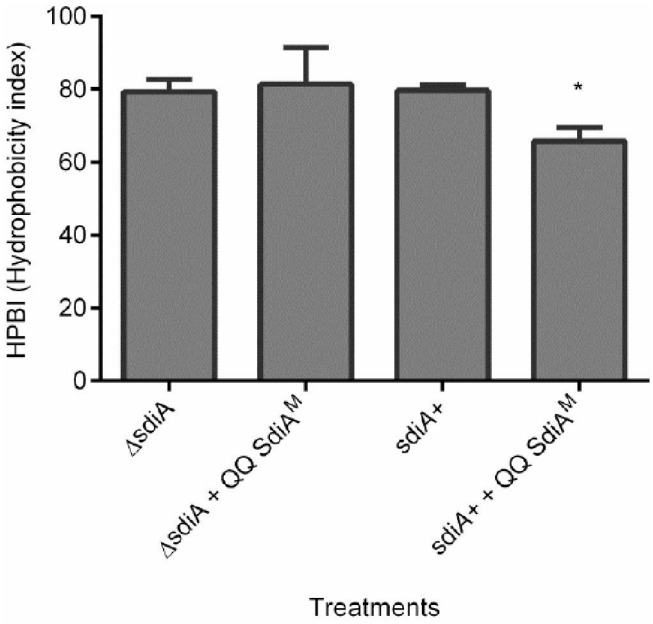

Figure 8 : Effect of QQ SdiAM on the mean Hydrophobicity index (HPBI) of EHEC O157:H7 strains. Error bars represent the significant differences between the groups (P<0.05)

The treated sdiA+ strain shows almost 50% decrease in protein expression, very comparable with the mutant strain levels of expression of FtsZ proteins, as seen in Figure 13, from the results of SDS PAGE. CLSM data generated using COMSTAT, including thickness, roughness coefficient, biomass and number of colonies of adherent cells is represented in Figure 14. The biomass and thickness show a marked decrease in the case of sdiA+ strain. QQ SdiA^M, at MBEC value showed cell viability of nearly 90% in HEp-2 cells. At 100 times MBEC, the cell viability reduced to nearly

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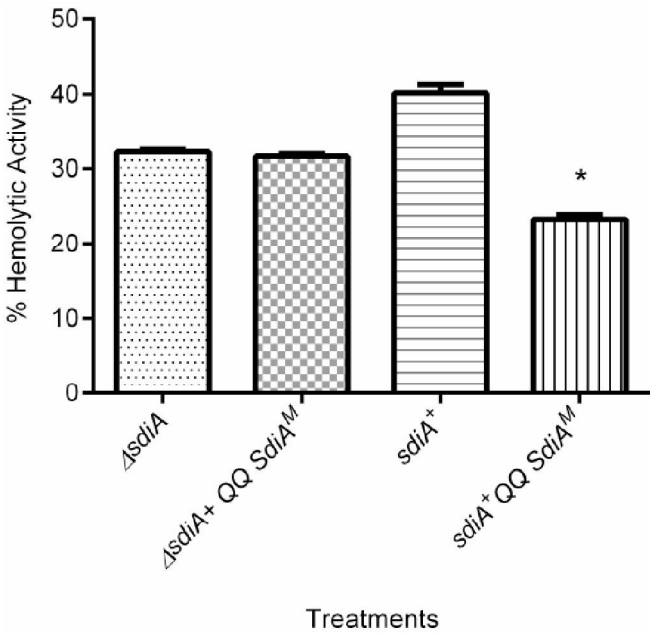

Figure 9 : Effect of MBEC of QQ SdiAM on % hemolytic activity of EHEC O157:H7 strains. Error bars represent the significant differences between the groups (P<0.05)

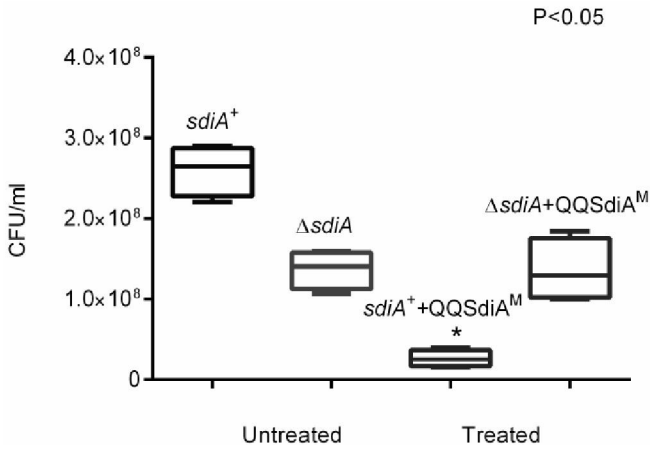

Figure 10 : Effect of QQ SdiAM on the acid resistance of EHEC O157: H7 strains

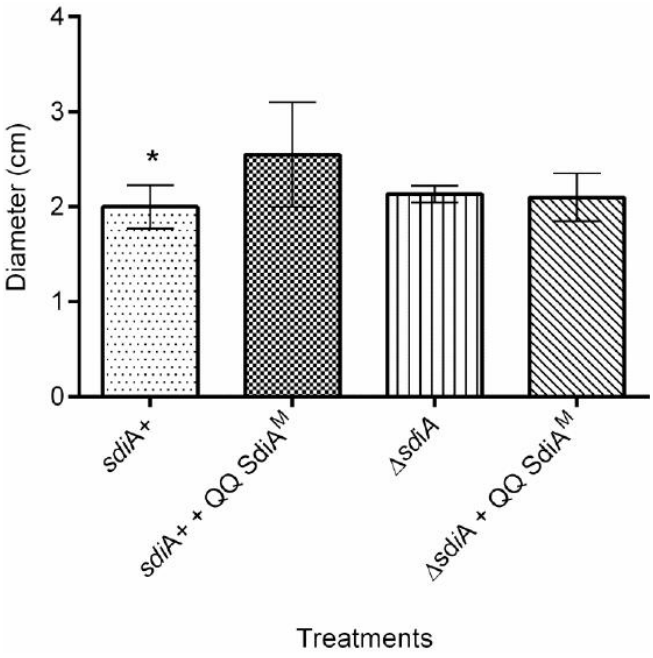

Figure 11 : Motility of EHECO157:H7 strains treated with QQ SdiAM

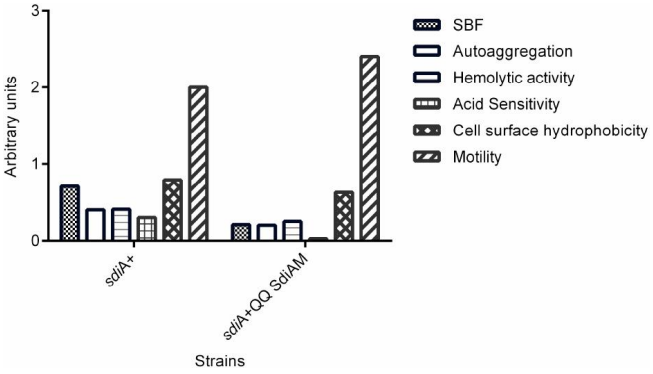

Figure 12 : A comparative study between the treated and untreated *sdiA*+ strain, show that the mechanism of action of QQ SdiAM is through SdiA

84%, which is not a very appreciable decrease. This is represented in Figure 15.

DISCUSSION

The preliminary growth studies showed that there were no major differences in the growth and final cell density of the 2 strains. This permits us to perform the various biochemical assays in a similar manner for both strains; it also shows that sdiA does not affect the growth characteristics of EHEC O157:H7. Initial biofilm formation assays predict that the maximum biofilm formation stage is attained at the 18th hour after incubation (late exponential phase), and there is a decline in the biofilm formation at the 24th hour. The results also indicate that the sdiA+ strain forms a stronger biofilm when compared to the mutant strain, proving that SdiA is involved in biofilm formation. This could probably be by activation of different genes that are involved in establishment of biofilms during the course of pathogenesis.

To determine the MBEC value of QQ SdiA^M, the tube and plate assays were performed. The least OD value and lowest CFU/ml were observed for a concentration of 1 µg/ml of QQ SdiAM. This value was fixed as MBEC value, and taken as the standard concentration in all the experiments that followed. The de-

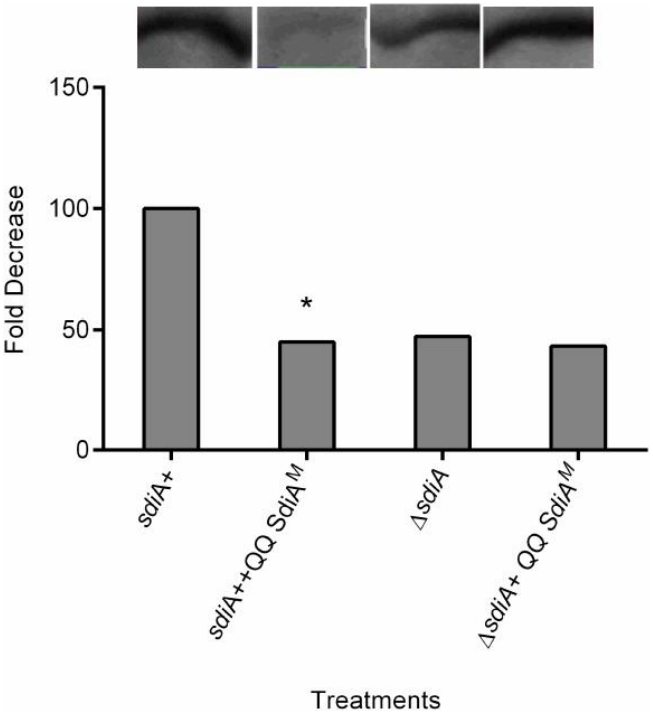

Figure 13 : The fold decrease of protein expression in EHEC O157:H7 strains, with reference to *sdiA*+

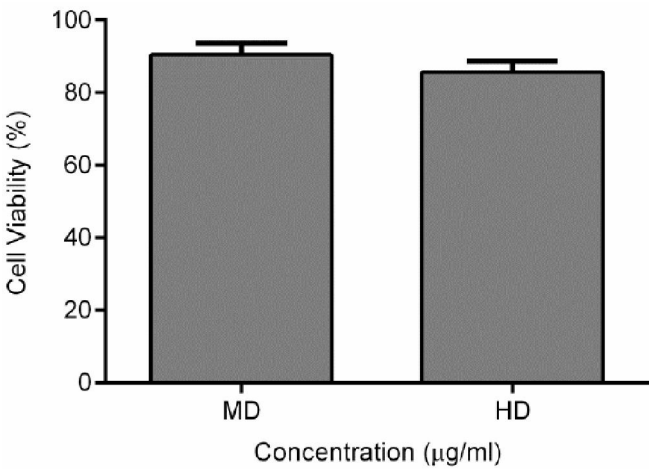

Figure 15 : Effect of QQ SdiAM (MBEC dose (MD-1 ìg/ml); High Dose (HD-100 ìg/ml) on % Cell Viability (%) of HEp-2 cells

crease in biofilm formation in the MBEC assay was apparent in the sdiA+ strain when compared to the mutant strain. Clearly, this means that QQ SdiA^M must act through SdiA in some manner. To validate this hypothesis, biochemical assays were performed, that

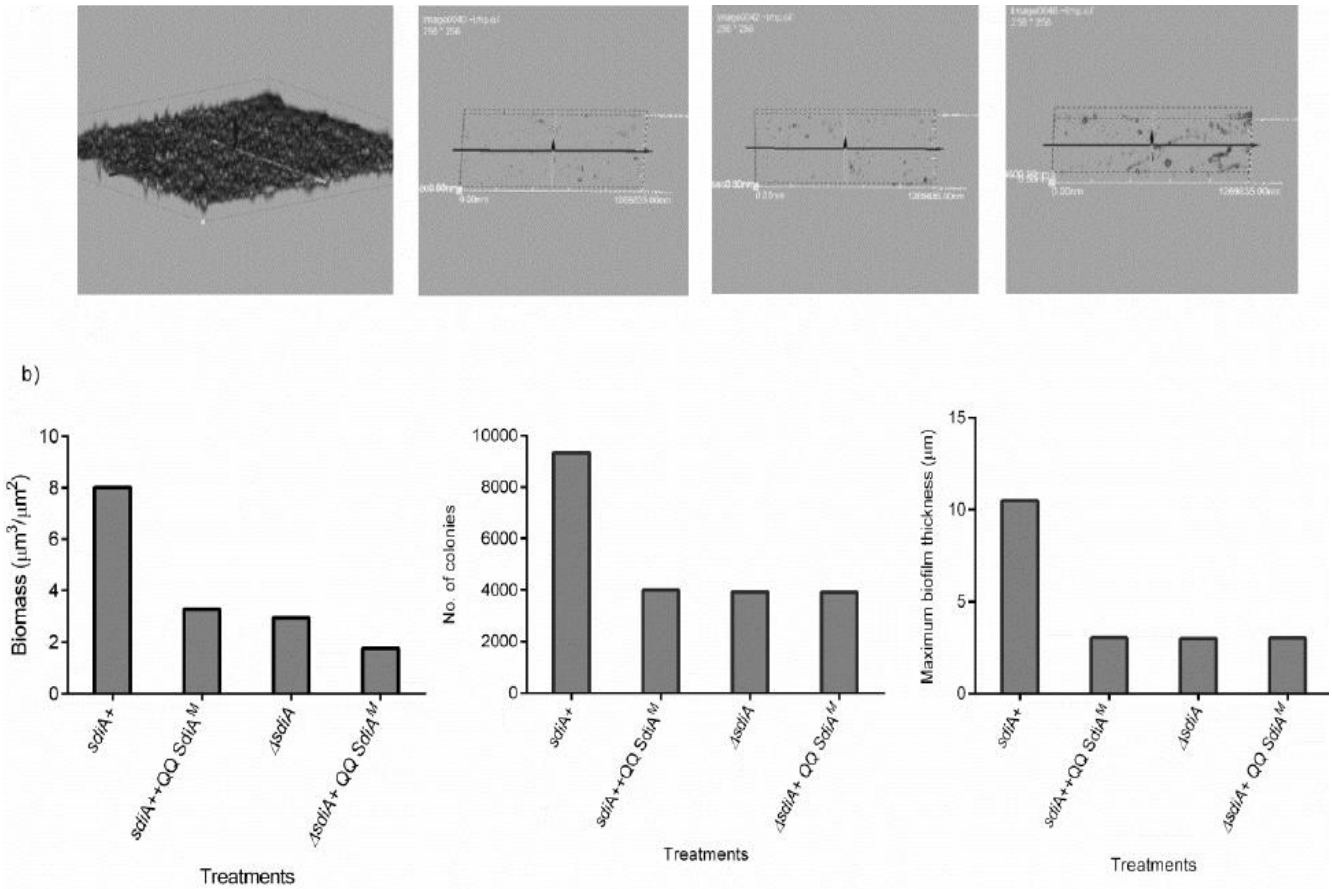

Figure 14 a) The Z plane CLSM images for EHEC O157:H7 strains treated with QQ SdiAM. b) The effect of QQ SdiAM on the biofilm parameters of EHEC O157:H7 treatments

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would test the effect of the drug on both the strains, with and without addition of the drug. Biofilm formation assay, when repeated with the addition of drug, showed a marked decrease at the late exponential phase, suggesting that QQ SdiA^M decreases the biofilm after its activation and keeps it at a low level of expression. The results of autoaggregation assay and cell surface Hydrophobicity indicate that QQ SdiA^M acts through SdiA, decreasing the %AA and %HI, and since both these factors are important in biofilm formation, a decrease in sdiA+ strain shows that QQ SdiAM is an antibiofilm compound. The results of hemolysin assay indicate that the decrease in % lysis of the sdiA+ strain could be due to the reason that sdiA activates the hly operon in plasmid pO157 of EHECO157:H7. By binding to SdiA, QQ SdiA^M could act as an SdiA antagonist and prevent its normal functions, thus reducing the hemolytic character of EHEC strains. An increase in acid sensitivity of sdiA+ strain with the addition of QQ SdiA^M showed that wild type cells are naturally resistant to acid, probably through SdiA. This correlates with the findings of … increase in motility suggests that SdiA naturally represses the mot operon, and that addition of drug prevents this repression, thereby increasing motility of sdiA+ strain, but not the mutant strain. Figure 12 summarizes these results appropriately.

FtsZ proteins are involved in the cell division of *E.coli* cells. It is possible that these proteins are under the control of SdiA. The 51% fold decrease in protein expression in sdiA+ strain, indicated both that the compound acts through SdiA and that SdiA is involved in over expression of FtsZ. Meanwhile, the mutant strains both showed 51 $\pm$ 3% protein expressions when compared to the sdiA+ control strain, again showing that SdiA is important in FtsZ expression.

The confocal analysis generated results showing a marked decrease in the biomass, maximum thickness and roughness coefficient. The number of colonies at the substrate has also decreased upon addition of QQ SdiAM in sdiA+ strain. Interestingly, the mutant strain showed no such decrease upon addition of QQ SdiA^M, infact a slight increase was observed in biomass. These overall results indicate quantitatively that important biofilm parameters do get affected upon addition of QQ SdiA^M, again proving its efficacy as an SdiA antagonist.

These results, coupled with the fact that housekeeping gene remains unaffected, shows that SdiA activates certain sets of genes involved in virulence and biofilm formation, and QQ SdiA^M, acts only through SdiA. Furthermore its low level of cytotoxicity to the HEp-2 cells makes it an ideal candidate as a potent drug molecule to cure EHEC O157:H7 infections, particularly HUS.

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