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Original Article

In vitro Biofilm Formation Ability of Clinical Isolates of *Salmonella enterica* Serovars Typhi and Paratyphi

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In the present study the ability of clinical isolates of *Salmonella enterica* serovars Typhi (n = 30) and Paratyphi A (n = 11) to form biofilm on abiotic surface was investigated. All isolates were found capable of biofilm formation in a microtitre plate assay. Upon optimization of biofilm formation by the test isolates, Adherence test medium (ATM) was found to be the best medium for biofilm formation by both *S. enterica* serovars Typhi and Paratyphi. Growth was optimized by incubation at 37°C in an orbital shaker set at 150 rpm for 48-72 hours. Biofilms were best detected when washed with PBS (1X), stained with crystal violet (1%) and subsequently washed with acetone (33%). Optical density (OD) readings were better correlated with growth at 570 nm when compared to 600 nm. Of the 28 *Salmonella* Typhi isolates, 17 (61%) were very strong biofilm producers, 8 (29%) were strong biofilm producers and 3 (11%) were moderate biofilm producers. On the other hand, out of 13 *S.* Paratyphi, 9 (69%) were very strong biofilm producers, 3 (23%) were strong biofilm formers and 1 (8%) was a moderate biofilm producer. None of them were weak biofilm producers. The present study raises concern from a public health point of view because the ability of the clinical isolates to form biofilm would indicate their ability of being transmitted from abiotic surface to uninfected host giving rise to disease.

Keywords: Biofilm, *Salmonella enterica* Typhi, Paratyphi, Planktonic

Introduction

Salmonella spp. are enteric pathogens notable for their ability to cause a range of diseases including gastroenteritis, septicaemia, osteomyelitis, pneumonia, meningitis, and arthritis[1]. The food borne pathogen *Salmonella enterica* and various members of the family *Enterobacteriaceae* are able to form biofilm on different biotic and abiotic surfaces[2-3]. A bioûlm is a group of microorganisms that attach to each other and to a biotic or abiotic surface, resulting in stability and protection from environmental factors mediated in part by a self-initiated exopolysaccharide (EPS) matrix[4]. Biofilms can become a persistent source of contamination[5] with increased ability to colonize and survive in a harsh condition[6]. The formation of biofilms involve multiple processes including initial surface attachment, monolayer formation, migration to form multilayered microcolonies, production of extracellular matrix and biofilm maturation with a three dimensional architecture[7]. A small number of bacterial cells adhere to the surface, a process facilitated by bacterial motility. Cells that attach irreversibly to the surface divide, forming microcolonies, that produce extracellular polymeric substances (EPS), primarily polysaccharides[8]. The EPS attaches the cells to the surface and stabilizes the colonies. With time, attached bacteria from the biofilm detach and disperse in order to survive and colonize new niches[9].

Understanding the formation of biofilms is important for their control. Biofilms are less susceptible to antimicrobials than are planktonic cells[10]. Bacteria within a biofilm are more resistant to environmental factors because of limited availability of key nutrients[5,11] and owing to the extracellular matrix[8]. Within a biofilm there is reduced diffusion, physiological changes due to reduced growth rates and production of enzymes which degrade antimicrobial substances[8] leading to increased resistance. Biofilms are a concern in the food industry as they can lead to illness, disease outbreaks with subsequent economic losses[12]. In the medical field, bacterial biofilms are worrying concerns because they can occur on the surfaces of medical devices and on tissue surfaces within compromised organs[13]. Biofilms grow similarly in the environment and in industrial systems[14]. In the present study, the ability of clinical isolates of *Salmonella enterica* serovar Typhi and Paratyphi to form biofilm *in vitro* was investigated. To our knowledge this constitutes the first world-wide report of the biofilm forming ability by clinical *Salmonella enterica* serovar Paratyphi. Previous reports on biofilm-forming *S. enterica* serovar Paratyphi were based on reference bacteria rather than on clinical isolates.

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Materials and Methods

Bacterial isolates

Twenty-eight *Salmonella enteric* serovar Typhi and 13 *S. enterica* serovar Paratyphi clinical isolates obtained from a hospital in Dhaka City, Bangladesh were used in this study. The isolates were confirmed by biochemical and serological tests.

Optimization of incubation conditions for maximum biofilm formation

(i) Media optimization

In spite of the fact that one earlier report has suggested the use of adherence test medium (ATM) for *Salmonella enterica* serovar Typhi[15] a number of media were tested for their ability to support maximum biofilm formation by the test strains. This was deemed necessary as strain variations may cause differences in requirement. The media used in this study included tryptic soy broth (TSB), **Mueller-Hinton broth** (MHB), Luria-Bertani broth (LB), Luria-Bertani broth with 5 g/l D-glucose (LB + glucose) and adherence test media (ATM).

(ii) Incubation condition optimization

In all conditions of optimization, incubation temperature of 37°C was used. Both static and shaker incubators were used. In shaker, two different shaking conditions, *viz*., 120 and 150 rpm were used. Time of incubation was also varied and biofilm production was observed after 24, 48 and 72 h incubation.

(iii) Biofilm staining and washing condition optimization

Non-adherent cells were washed with two different washing solution, *viz*., double distilled H_2O and 1X PBS (phosphate buffer saline). Biofilm was stained with crystal violet at two different concentrations of 0.03 and 1% for 30 min. To detach biofilm from wall of the tube, two different solutions were used, *viz*., 80:20 = ethanol:acetone and 33% acetic acid, which were applied for 15 min.

(iv) Wave length and absorbance optimization

In case of 80:20 = ethanol:acetone solution absorbance was taken at 600 nm. In case of 33% acetic acid solution absorbance took at 570 nm. Absorbance was obtained after 24, 48 and 72 h incubation in ELISA plate reader.

Bacteriological medium

Adherence test medium (ATM) with slight modifications was adapted following optimization of biofilm formation and medium was prepared as described earlier[15]. This medium contained 60 m*M* NaCl, 20 m*M* KCl, 111 m*M* glucose and 30 m*M* $NaHCO_3$. The pH was adjusted to 8.4. To this a supplement containing NH_4Cl (20 m*M*), Na_2HPO_4 (40 m*M*), $(NH_4)H_2PO_4$ (50 m*M*), $CaCl_2$ (999 µ*M*), $MgCl_2$ (980 µ*M*), $FeCl_3$ (86 µ*M*) and Na_2SO_4 (40 m*M*) was added separately.

Biofilm assay

The ability of the bacterial strains to form bioûlms in polystyrene (PS) microtitre plates was evaluated by using the method described elsewhere[16] with some modifications. Colonies from an overnight plate was grown in adherence test medium (ATM) at 37°C in an orbital shaker (120 rpm) to obtain a MacFarland standard of 0.5 equivalents of cells. From this, 200 µl of bacterial suspension was transferred into a well of a sterile 96-well PS microtitre plate and the plate was then incubated for 48 hours at 37°C, 120 rpm to allow biofilm formation. Planktonic bacteria were removed and each well was washed thrice with phosphate buffered saline (dissolving 8 g of NaCl, 0.2 g of KCl, 1.44 g of Na_2HPO_4, 0.24 g of KH_2PO_4 in 1 l distilled H_2O and pH adjusted to 7.4) to remove loosely attached cells. Subsequently, 200 µl of 1% (wt/vol) crystal violet solution (Sigma-Aldrich/ Life Science Chemilab SA, Athens, Greece) was added into each well, and the plate was then incubated for 30 min at room temperature. After being washed three times with 200 µl of PBS to remove excess stain, the crystal violet was solubilised in 200 µl of 33% acetate solution. Dye absorbance at 570 nm was measured using a microtitre plate reader (Sunrise, Tecan, Männedorf, Switzerland). Each experiment (isolate) was done in triplicate wells. *Salmonella enteric* serovar Typhimurium ATCC 14028 was used as a positive control for biofilm formation and sterile ATM were used as negative controls. When the absorbance values of the crystal violet bound to the sample bacteria was shown at least twice that of the control it was considered as positive result for bioûlm formation. Based on the binding of crystal violet by the biofilm forming bacteria the isolates were categorized as very strong (VS) biofilm producers, strong (S) biofilm producers and moderate (M) biofilm producers using a modified convention described earlier[17].

Results

Optimized incubation conditions for biofilm formation

Following optimization, best biofilm production was obtained in ATM medium at 37°C in 150 rpm shaker after an incubation time of 48 h. Among the two washing solutions used, 1X PBS was the better washing solution. For staining of biofilm, 1% crystal violet worked better than the lower concentration of 0.03% when used for 30 min. As detachment solution, 33% acetic acid solution worked better than ethanol:acetone = 80:20. Absorbance was taken at 570 nm after 48 h of incubation. Figure 1 depicts the OD_{600} values when using the optimized conditions for biofilm formation. The results are mean of four readings and are expressed as mean ± 1 standard deviation.

Biofilm formation by the S. enterica Typhi *and Paratyphi isolates*

All isolates of *S. enterica* Typhi and Paratyphi were tested in triplicate were found to be capable of forming biofilm to different extents. The degree of crystal violet retention is an indication of the number of bacteria in the biofilm which bind to crystal violet. Hence, the absorbance at 570 nm correlates positively with the number of biofilm producer. The cut-off OD was taken as two standard deviations above the mean value of negative control. Figure 2 represents the mean OD values of the crystal violet retained by the *Salmonella* Typhi isolates and the controls and Figure 3 depicts that retained by *S. enterica* Paratyphi.

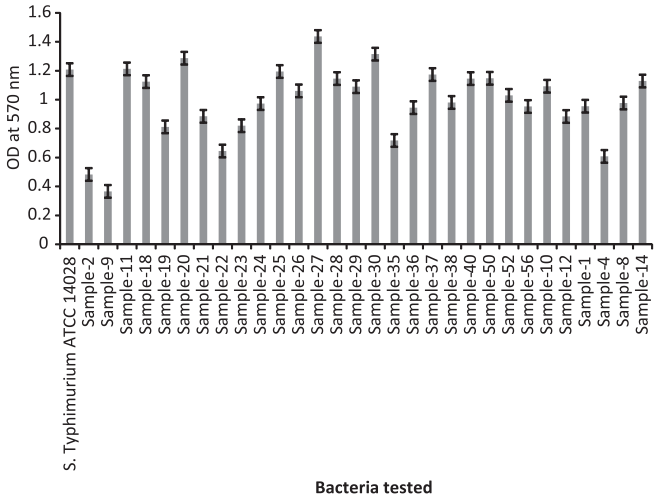

Figure 1. *Biofilm formation under optimized condition (ATM medium incubated at 37°C in an orbital shaker adjusted to 150 rpm and incubated for 48-72 hours. Biofilms were washed with PBS, 1X, stained with crystal violet, 1% and washed with acetone, 33%). All experiments were carried out four times. Results are mean* OD_{570} *± SD.*

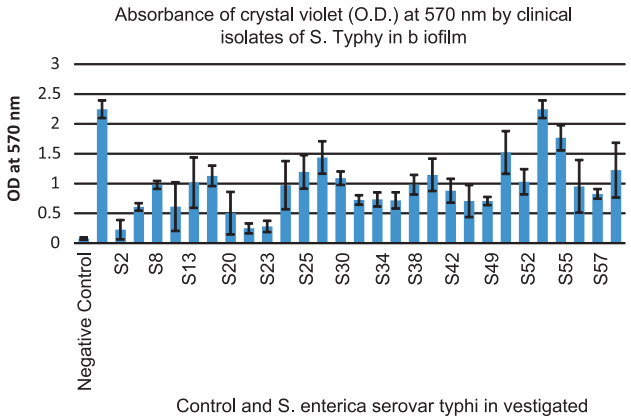

Figure 2. *Absorbance of crystal violet* OD_{570} *by clinical isolates of Salmonella enterica serovar Typhi biofilms in modified ATM. Results are mean of three readings ± 2 SD.*

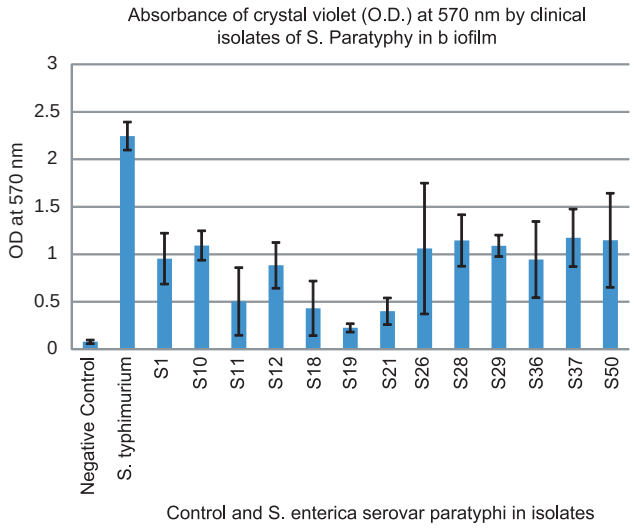

Figure 3. *Absorbance of crystal violet* OD_{570} *by clinical isolates of Salmonella enterica serovar Paratyphi biofilms in modified ATM. Results are mean of three readings ± 2 SD.*

Categorization of the isolates on the basis of strength of biofilm formation

The isolates were classified as follows: non-producing, weak, moderate, and strong-producing, based on the following optical density (OD) average values: OD (Isolate) d" OD (Control) = Non-biofilm-producer; OD (Control) d" OD (Isolate) d" 2 OD (Control) = Weak biofilm producer; 2 OD (Control) d" OD (Isolate) d" 4 OD (Control) = Moderate biofilm producer; 4 OD (Control) d" OD (Isolate) = Strong biofilm producer. We modified these interpretive criteria by adding yet another category, which we described as 8 OD (Control) d" OD (Isolate) = Very strong biofilm producer. In this case, the category 'strong biofilm producer' was described as 4 OD (Control) d" OD (Isolate) d" 8 OD (Control) = Strong biofilm producer. According to this categorization, of the 28 *S. enterica* Typhi isolates, 17 (61%) were very strong (VS) biofilm producers, 8 (29%) were strong (S) biofilm producers and 3 (11%) were moderate (M) biofilm producers (Table 1). On the other hand, 9 (69%) of the *S. enterica* Paratyphi were very strong (VS) biofilm producers, 3 (23%) were strong (S) biofilm formers and 1 (8%) was a moderate (M) biofilm producer.

Table 1. *Categorization of the Salmonella enterica Typhi and S. Paratyphi isolates in terms of biofilm forming ability*

Isolate	Biofilm category	OD control/OD sample
Salmonella Typhi isolates (n = 28)		
S. Typhimurium	VS	8 OD (Control) d" OD (Isolate)
S8, 13, 14, 24, 25, 27, 30, 38, 40, 42, 51, 52, 54, 55, 56, 57, 58	VS	8 OD (Control) d" OD (Isolate)
S4, 9, 20, 32, 34, 35, 45 and 49	S	4 OD (Control) d" OD (Isolate) d" 8 OD (Control)
S2, 22, 23	M	2 OD (Control) d" OD (Isolate) d" 4 OD (control)
Salmonella Paratyphi isolates (n = 13)		
S1, 10, 12, 26, 28, 29, 36, 37, 50	VS	8 OD (Control) d" OD (Isolate)
S11, 18, 21	S	4 OD (Control) d" OD (Isolate) d" 8 OD (Control)
S19	M	2 OD (Control) d" OD (Isolate) d" 4 OD (Control)

VS = Very strong; S = Strong; M = Moderate.

Microscopy of a sample biofilm

A sample of the biofilm was observed under the light microscope (100x) following crystal violet staining. The micrograph indicated clumps of biofilm bacteria (Figure 4). As negative control, blank medium was used instead of bacterial culture. The micrograph which reflected a blank view has not been shown here.

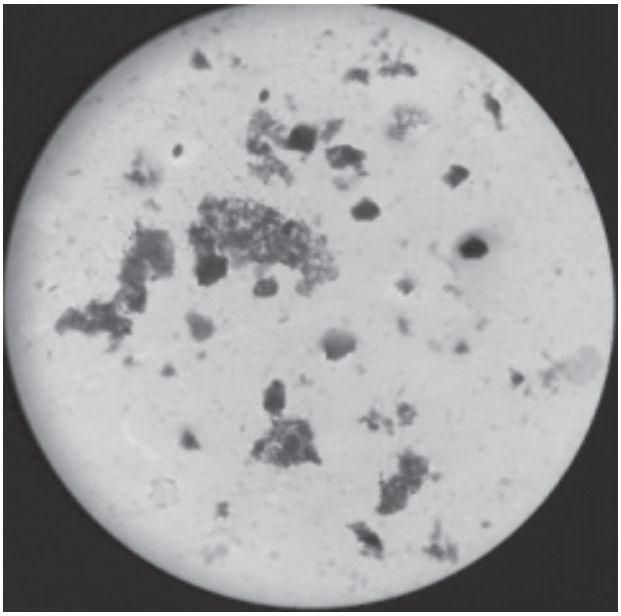

Figure 4. *Light micrograph of a representative biofilm forming Salmonella enterica serovar Typhi.*

Discussion

*Salmonella*e are recognized worldwide as major zoonotic pathogens for both humans and animals. Most microorganisms persist in a biofilm ecosystem and not as free-floating organisms. The ability of *Salmonella* to attach to food surfaces was the first published report on food-borne bacterial biofilms[18].

In the present study, all investigated *S. enterica* Typhi and Paratyphi isolates were found to be capable of forming biofilms. Different culture media, incubation conditions solutions were used for biofilm washing and detachment. The best biofilm formation for both *S. enterica* Typhi and Paratyphi was observed in ATM medium at 37°C under vigorous aeration (150 rpm). This finding is similar to that of Raza *et al*.[15] who modified ATM medium slightly for optimum biofilm formation by *S. enterica* Typhi only.

There are several reports that describe the ability of *Salmonella* to form biofilms on abiotic surfaces outside the host, such as stainless steel[19], plastic[20], rubber[21], glass[2], cement[22], marble and granite[14]. All these surfaces are commonly encountered in farms, slaughter houses, food industries and kitchens which raise the risk for public health. It is strongly believed that the ability of *Salmonella* to form biofilms on inanimate surfaces contributes to its survival and persistence in non-host environments and its transmission to new hosts. However, adhesion by *Salmonella* is strain-dependent and probably influenced by surface structures, such as cell wall and membrane proteins, fimbriae, flagella and polysaccharides[19,23-24] has reported on the ability of reference *S. enterica* Typhi and Paratyphi isolates to form biofilm in microtitre plate.

Conclusion

The significance of the present study lies in the fact that all isolates were clinical in origin and most of them were very strong biofilm producers. This raises the chance of formation of biofilms by clinical *Salmonella enterica* serovars Typhi and Paratyphi on abiotic surfaces, a condition which is of public health significance since attached bacteria on commonly used plastic surfaces can aid transmission to uninfected hosts and gives rise to disease. Persistence of such biofilm bacteria on abiotic surfaces may form the basis of future studies.

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