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Effect of biofilm formation on the excretion of *Salmonella enterica* serovar Typhi in feces

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SUMMARY

Objectives: We hypothesized that *Salmonella enterica* serovar Typhi (*S.* Typhi) with higher biofilm and capsule production capability are more able to survive continuously in typhoid patients/carriers, with subsequent prolonged shedding in feces.

Methods: Bacterial cell release from biofilm (produced in vitro and confirmed by specific staining and electron microscopy) and comparative cytotoxicity were studied on Caco2 cells. Functionality of the biofilm diffusion barrier was tested against ciprofloxacin. Biofilm production was graded and semi-quantified as −, +, ++, +++, and ++++.

Results: Out of 30 isolates, 23 produced biofilm. The average post-treatment detection of *S.* Typhi in blood was 7–13 days and in stool was 13–32 days. A fall in cell count from 10^4 to approximately 10^1 over the course of 3 days as compared to total elimination of planktonic cells in 16 h after ciprofloxacin application substantiated the protective role of biofilm. Lactic dehydrogenase release ranged from 38% in non-biofilm producers to 97% in the highest biofilm producers, indicating increased pathogenic behavior.

Conclusions: The period of *S.* Typhi clearance from typhoid patients after recovery was found to be directly related to biofilm production capability.

1. Introduction

Bacterial biofilms are the predominant mode of bacterial growth, reflected in the observation that approximately 80% of all bacterial infections are related to biofilms.[1,2] Biofilms are defined as structured communities of bacterial cells enclosed in a self-produced polymeric matrix adherent to inert or living surfaces.[3–5]

Salmonella enterica serovar Typhi (*S.* Typhi), the causative agent of typhoid in humans, is also capable of producing biofilms; this contributes to its resistance and persistence in the host. *S.* Typhi is transmitted through the fecal–oral route by contaminated water and food. Typhoid is communicable for as long as the infected person is capable of excreting bacteria in stool. These bacteria usually disappear from the stool about a week after symptoms of illness have resolved. However, a percentage of these infections can result in asymptomatic carriage of salmonellae, possibly due to the formation of biofilms as a mechanism that contributes to the development of the carrier state.[6]

Bacteria in biofilms are generally considered well protected against environmental stresses, antibiotics,[7] disinfectants, and the host immune system,[8] and as a consequence are extremely difficult to eradicate.[9] Planktonic *Salmonella* populations are found to be sensitive to different antibiotics as compared to biofilms. It is reported that *Salmonella enterica* serovar Typhimurium biofilms pre-formed on microplates are up to 2000-fold more resistant to ciprofloxacin as compared to planktonic cells.[10] This is particularly concerning, as ciprofloxacin is commonly used to treat *Salmonella* infections.[11]

Traditionally, the ability of *S.* Typhi to cause disease and to induce a protective immune response is attributed to possession of a capsule that is polysaccharide in nature. Yet it is also well known that *S.* Typhi can cause disease in the absence of capsule.[12,13] As biofilm has a protective role similar to capsule, we hypothesized that its presence may have a shielding role and be a basis for longer survival in the body, thus substantiating the carrier status.

This study was designed to evaluate the possible role of biofilm produced by *S.* Typhi on delayed clearance of bacteria (extended carrier state) from the body in association with the presence of the outer capsular polysaccharide, and the comparative efficacy of anti-typhoidal drugs, especially ciprofloxacin, against planktonic and biofilm phase bacteria.

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2. Materials and methods

2.1. Clinical samples

Clinically suspected cases of typhoid (both sexes; age range 8–55 years) with a fever of 3–20 days duration and most of the following symptoms were studied: enlarged spleen, headache, rose spots, malaise, abdominal discomfort, lethargy, constipation followed by diarrhea, fatigue, delirium, and agitation. One hundred patients who were PCR-positive (targeting the *fli*C gene) and were due to receive standard typhoid treatment were included. Clinical specimens were collected on the same day or within 1–2 days after the first consultation. Series of blood and stool samples were collected (twice a week) from each patient until the PCR became negative for at least two consecutive collections. Blood samples were collected simultaneously in potassium EDTA (20 mM) BD Vacutainer for PCR and in sterilized tryptic soy broth (TSB) for blood culture (1:8), while stool samples were collected in sterile containers containing glycerol saline buffer (dipotassium phosphate 22.7 mM (3.1 g/l), monopotassium phosphate 7 mM (1 g/l), phenol red (0.003 g/l), sodium chloride 72 mM (4.2 g/l)). Samples that were blood culture-positive (28 out of 100) and identified as *S*. Typhi by conventional biochemical and molecular methods,[14,15] were selected for further study. These isolates were subcultured in TSB overnight, and tested for Vi antigen by corresponding antiserum (Bio-Stat, UK); aliquots were preserved in 20% glycerol and stored at −20 °C until further use. When required, an aliquot of the stored *S*. Typhi isolate was revived in TSB for 24 h at 37 °C.

2.2. Polymerase chain reaction (PCR)

DNA was extracted from blood as described previously.[16] Briefly, 1 ml of blood containing 20 mM potassium EDTA as anticoagulant was centrifuged at 10 000 rpm (Sorvall Legend RT) for 5 min. Plasma was separated for serology. The pellet was resuspended in 1 ml of lysis buffer (0.2% Triton X-100 in Tris–HCl (pH 8.0)). The mixture was gently aspirated several times to encourage efficient hemolysis. The tube was centrifuged at 12 000 rpm (Sorvall Legend RT) for 6 min, the supernatant was discarded, and the procedure was repeated. The pellet was washed with distilled water. The supernatant was removed, and the pellet was subsequently resuspended in 20–30 μl of distilled water. The tubes were sealed and then sterilized in boiling water for 20 min. Extraction of bacterial DNA from fecal samples was performed according to Frankel et al.[17]

Molecular detection of *S*. Typhi was done targeting the *fli*C gene by regular primers ST1 5′-TATGCCGCTACATATGATGAG-3′ and ST2 5′-TTAACGCAGTAAAGAGAG-3′, and nested primers ST3 5′-ACTGCTAAAACCACTACT-3′ and ST4 5′-TGGAGACTTCGGTCGCGTAG-3′;[15] conditions have been described previously.[18] The *via*B operon, and type IV B pili, which are essential for capsule formation and bacterial attachment, were detected in all *S*. Typhi isolates by targeting the *tvi*A and *pil*S genes, respectively.[19] Two reference strains NIB25 and NIB38,[19] were used as negative and positive controls, respectively, for both the *via*B operon and type IV B pili. Oligonucleotides and enzymes used in the study were supplied by Fermentas (Maryland, USA). Amplicons were separated on a 2% agarose gel at 100 V for 60 min and photographed using Gel DocTM-XR imaging system (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

2.3. Biofilm production by S. Typhi

After detection and confirmation of *S*. Typhi isolates and evaluation of their Vi status with PCR, we followed the reported methodology for the production of biofilms.[20] However, as adherence test medium (ATM) failed to produce biofilm, we used modified biofilm production medium, which was optimized to contain 60 mM NaCl, 20 mM KCl, 110 mM glucose, 30 mM Na_2HCO_3, 20 mM NH_4Cl, 40 mM K_2HPO_4, 50 mM $(NH_4)H_2PO_4$, 1 mM $CaCl_2$, 980 μM $MgCl_2$, 86 μM $FeCl_3$, and 40 mM Na_2SO_4. The suspension was placed in grease-free sterilized sealed test tubes in triplicate and incubated with mild shaking (170 rpm) at 37 °C for 24 h. For semi-quantitative grading we developed a reference that subdivided the *S*. Typhi isolates into five categories, ranging from no biofilm production (−) to maximum biofilm production (++++).

2.4. Crystal violet staining of biofilm

Crystal violet staining of biofilm was done following the methodology described elsewhere.[21] Briefly, planktonic phase cells were aspirated and biofilm ring was washed with a continuous spray of 1× phosphate buffered saline (PBS; pH 6.8) and incubated at room temperature for 1 h to fix the cells. Crystal violet (1% in isopropanol–methanol–1× PBS; 1:1:18) was poured into each test tube. Test tubes were incubated for 15 min at room temperature and washed thoroughly with 1× PBS (pH 6.8) until the buffer ran clear. Biofilm was then immersed in 33% acetic acid to extract the dye. Dye retained by the bacterial cells was measured at 570 nm. For quantification, a standard graph of crystal violet in 33% acetic acid was made. Dye retained by the bacterial cells was measured at 570 nm in batches of six.

2.5. Transmission electron microscopy (TEM) of biofilm

Samples from the interface, planktonic phase, and TSB were analyzed using TEM (Jeol 1010, Japan). For micro-encapsulation method, agar (3%) blocks with biofilm samples were prepared, thinly sliced, and studied under TEM. Direct analysis of biofilm matrix on AEI carbon-coated grids was done. Bacterial biofilm suspension was placed onto the grid and the bacteria were allowed to adhere for 2 min and then fixed for 1 min with 1.5% glutaraldehyde in sodium cacodylate buffer (100 mM, pH 7.4). The grids were rinsed twice with water and negatively stained with 0.75% (wt/vol) uranyl acetate (pH 6.4) for 1 min. The grids were drained and subjected to microscopic studies.

2.6. Antibiotic susceptibility assay

Four commonly used antibiotics for typhoid were employed to compare susceptibility patterns of biofilm resident and planktonic phase bacteria. Pieces of biofilm were cultured in 5 ml TSB overnight, whereas for planktonic bacteria, 50 μl of inoculated medium from the same tube was added to 5 ml of TSB and incubated overnight. The antimicrobial susceptibility patterns were determined as per the Clinical and Laboratory Standards Institute (CLSI) recommendations,[22] using the following commercial antimicrobial disks (HiMedia, India): chloramphenicol (30 μg), ampicillin (10 μg), ciprofloxacin (5 μg), and trimethoprim (30 μg).

2.7. Ciprofloxacin penetration assay

Biofilm was exposed to 1 μg/ml of ciprofloxacin. Planktonic phase cells were also transferred, essentially without dilution, into fresh antibiotic-containing growth medium. Colony count experiments were performed in parallel. For the penetration assay, biofilm produced was exposed to ciprofloxacin for specified time intervals of 4, 8, 12, 16, 20, 24, 36, 48, 60, and 72 h. After exposure to ciprofloxacin, the biofilm gummy material was used for colony count experiments.

Table 1
LDH release assay for cytotoxicity after exposure to ciprofloxacin (1 μg/ml)

	Cytotoxicity of escapers		Ciprofloxacin penetration
	LDH release ($A_{490/655}$)	% Cytotoxicity	% Bactericidal activity
Control (media)	0.16 ± 0.04		
Control (Triton X)	1.55 ± 0.31		
Control positive (free S. Typhi culture)	1.48 ± 0.40	Taken as 100%	100% after 16 h
After (h):			
4	1.11 ± 0.03	74.65	30
8	0.98 ± 0.23	60.95	40
12	0.81 ± 0.05	55.47	52
16	0.83 ± 0.02	56.84	60
20	0.75 ± 0.3	52.73	65
24	0.78 ± 0.04	47.94	63
36	0.71 ± 0.31	47.26	79
48	0.60 ± 0.41	39.04	85
60	0.48 ± 0.05	30.13	88
72	0.41 ± 0.08	27.39	94

LDH, lactate dehydrogenase.

2.8. Lactate dehydrogenase (LDH) assays

We used the increase in LDH release to show if *S.* Typhi cells in biofilm are more pathogenic than planktonic cells. Human colon epithelial cell line Caco2 was used for the assessment of LDH release.[23] Caco2 cells ATCC (Rockville, MD, USA) were grown in Dulbecco's modified Eagles medium (DMEM) as monolayers and trypsinized. Viability counts were done by trypan blue (0.4%) staining to assess the suitability for further experimentation.[23] Biofilm was produced in a 96-well plate, and 200 μl of Caco2 cell suspension was added for selected time periods (4, 8, 12, 16, 20, 24, 36, 48, 60, and 72 h, Table 1). The cell suspension was aspirated after a specified time and centrifuged (3000 × *g*, 5 min) to remove debris. A 0.1-ml aliquot was dispensed into a 96-well microtiter plate, and 0.1 ml/well of LDH substrate was added. Plates were read after 10 min of incubation at room temperature using a plate reader (Bio-Rad, Hercules, USA) at 490/655 nm. For the purpose of calculating cytotoxicity values, background LDH release from tissue culture cells was considered as low (media) control and Triton-X 100 (0.01%) treated cells as high control. The experiment was performed with high-grade biofilm producing *S.* Typhi isolates in batches of eight.

2.9. Statistical analysis

Analysis of variance was used to determine the differences among all four biofilm groups (high, medium, low, and non-biofilm producers). The Tukey test was applied to check the differences between each two of the biofilm groups, and the mean difference was considered as significant at the 0.05 level. Data were analyzed using statistical software SPSS version 16 (SPSS Inc., Chicago, IL, USA).

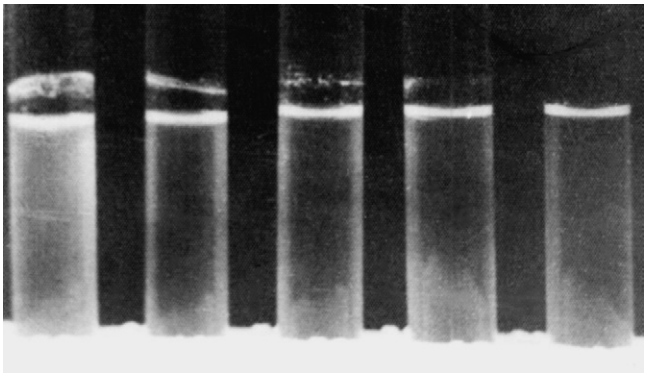

Figure 1. In vitro glass adherence test for *Salmonella* Typhi biofilm production. Biofilm production reference for *S.* Typhi isolates: 24 h growth in modified biofilm production medium at 170 rpm at 37 °C. Biofilm production was graded (from left to right) as ++++, +++, ++, +, and −, respectively.

3. Results

3.1. Biofilm production

Forty percent (12/30) of the isolates were able to produce a high level of biofilm (grade ++++ and +++), 16.7% (5/30) medium (++), 20% (6/30) low grade (+), and 23.3% (7/30) were unable to produce biofilm (Figure 1). These results included the reference strains.

3.2. Biofilm matrix analysis

Electron microscopy confirmed the presence of biofilm matrix (Figure 2). Cells were found to be embedded in the form of macrocolonies at the interface (Figure 2A) as compared to planktonic phase cells in the middle of the test tube (Figure 2B). The biofilm, which appeared as a slimy whitish gunk to the naked eye, was observed as multicellular communities attached by water channels that are represented by thread-like structures in TEM images. Without shaking no biofilm was produced; only aggregation in the middle of the test tube was observed. An increase in polysaccharide formation was observed after 24 h. Crystal violet staining confirmed the biofilm production.

3.3. Biofilm production phenomenon in relation to clearance of S. Typhi from the body

Post-treatment, the last day of *S.* Typhi detection in blood ranged from 10–15 days (mean 13.125 ± 1.96) in high biofilm producers to 7–15 days (mean 9.8 ± 3.27) in medium producers, 8–11 days (mean 8.88 ± 1.21) in low-grade producers, and 5–10 days (mean 6.85 ± 1.67) in non-biofilm producers. Similarly, the last day of detection of *S.* Typhi in feces had a mean value of 32.25 ± 12.78 days in high-grade biofilm producers, 23.6 ± 7.5 days in moderate biofilm producers, 16.51 ± 2.13 days in low biofilm producers, and 13.28 ± 2.81 days for non-biofilm producers, indicating a role of biofilm production in the carrier state.

Regarding days to detect the *S.* Typhi in blood, the comparison of high biofilm producers with low and non-biofilm producers showed a significant difference ($p = 0.04$ and $p < 0.001$ respectively), while the difference among all other biofilm groups was found to be non-significant ($p > 0.05$). In the case of detection from stool, only

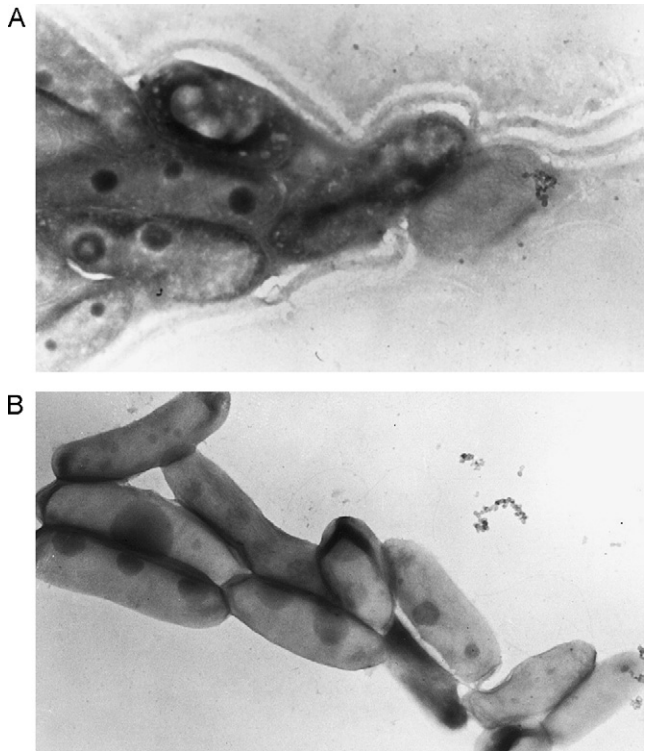

Figure 2. Transmission electron micrographs of biofilm (Magnification × 23 500). (A) Biofilm producing *Salmonella* Typhi. (B) Non-biofilm producers.

the difference between high and non-biofilm producers was found to be significant ($p = 0.008$), and the difference among all other biofilm groups was found to be non-significant ($p > 0.05$). Details are given in (Table 2).

3.4. Antibiotic susceptibility of biofilm and planktonic phase cells

Out of 30 isolates, 23 (76%) produced biofilm; 19 were found resistant to one or more anti-typhoid drugs, i.e., chloramphenicol (Cm), ampicillin (A), trimethoprim (T), and ciprofloxacin (C). Of the seven isolates that failed to produce biofilm, four were found sensitive to all four antibiotics (Table 2). No difference was found in the resistance patterns of the cells from biofilm matrix and planktonic phase when tested against all four antibiotics.

3.5. LDH assay for cytotoxicity

A marked difference in LDH release was observed between the two categories. More LDH release, 62% to 97%, was observed in isolates with a high-grade biofilm production level as compared to non-biofilm producers (i.e., 38% to 57%), showing that biofilm producers are more cytotoxic (Table 1). The LDH release assay was also used to study the continuous escape of bacteria from biofilm for different time intervals of 4 to 72 h. In the first 4 h, maximum cytotoxicity (~75%) was observed with more LDH release, which decreased with time but did not reach 0% in 72 h, showing the presence of cells (persisters) inside the biofilm (Table 1).

The LDH release from high biofilm producers was found to be significantly higher than in low and non-biofilm producers ($p < 0.001$). The difference between medium and non-biofilm producers was also found to be significant ($p = 0.008$), while the difference among all other biofilm groups was found to be non-significant ($p > 0.05$).

3.6. Ciprofloxacin penetration assay

Evidence for persisters was further strengthened with the ciprofloxacin penetration assay in which the drug was able to penetrate into the biofilm reducing the cell count from 10^4 to approximately 10^1 over the course of 3 days, although the free bacteria were totally killed after 16 h of exposure at 1 μg/ml. Penetration into biofilm was slow (killing almost 30% of cells in the first 4 h and up to 94% after 72 h) (Table 1).

3.7. Virulence status of biofilm producers and non-producers

Of the 30 isolates, seven failed to give any amplification for the *tvi*A gene. All the high-grade biofilm producers produced the desired amplicon; among medium and low biofilm producers 4/5 and 4/6 were *tvi*A-positive, respectively. The *pil*S was detected in all biofilm-producing isolates. The non-biofilm producers showed variable results; out of seven isolates, three were found positive for both *tvi*A and *pil*S, whereas four failed to give any amplification for both genes. Details are described in Table 2.

4. Discussion

Typhoid is communicable for as long as the infected person excretes *S.* Typhi in the feces. Despite major treatment and prevention efforts, millions of new typhoid infections occur worldwide each year. For a subset of infected individuals, *S.* Typhi colonizes systemically, mostly in the gall bladder, and remains long after symptoms subside, serving as a reservoir for the further spread of the disease.[24] The excretion in stool usually begins about a week after the onset of illness and continues through convalescence and for a variable period thereafter.[25]

Biofilm formation is likely to play a significant role in establishing long-term colonization, and bacterial cells are continuously shed for extended periods.[6] In this study, we tried to find a correlation between this carrier state and the biofilm production capability of isolates, if any. We found that shedding of *S.* Typhi in stool continued for a longer time in patients infected with high-grade biofilm producers. The maximum period for shedding of bacteria observed in this study was 50 days (average 32.25 days) post-infection in the case of high biofilm producers, and this was usually not more than 17 days (average 13.28 days) in the case of non-biofilm producers. The presence of biofilm in *S.* Typhi may thus be related to the length of the carrier state in a patient after recovery.

Although biofilm production prolonged the carrier state, it remains to be evaluated whether this was due to the physical protective effect or to the biofilm bacteria being more resistant as compared to planktonic phase bacteria. Recently, 194 *S. enterica* strains isolated from infected children were investigated for their ability to form biofilms on silicone disks; these were compared with corresponding planktonic forms for susceptibility to nine antimicrobial agents. About 56% of the strains were able to form biofilms.[26] The biofilms showed increased antimicrobial resistance to all antibiotics as compared to the planktonic bacteria, with the highest resistance rates for gentamicin (90%) and ampicillin (84%). Our findings also show that when the bacterial cells are detached from biofilm, they show similar drug resistance patterns to the planktonic phase cells. However, they were more cytotoxic as shown by increased LDH release from target Caco2 cells.

Real-time penetration of ciprofloxacin dropped the cell number from 10^4 to approximately 10^1 at 1 μg/ml, but it was not able to eliminate 100% of the cells and left the persisters intact. This finding is consistent with other reports regarding persisters.[10] Once the antibiotic level drops, the persisters may multiply, explaining the relapsing nature of biofilm infections.

Table 2
Individual characteristics of Salmonella Typhi isolates

No.	Isolate	Last day of blood PCR positive[a]	Last day of stool PCR positive[a]	Drug resistance pattern[b]	Biofilm visual grading	*tvi*A gene	*pil*S gene	LDH release
1	ST1275	15	30	Cm, T	+++	Present	Present	1.59
2	ST1389	13	15	A, T	++++	Present	Present	1.48
3	ST1594	12	40	A, Cm, T	++++	Present	Present	1.57
4	ST1403	14	33	A	++++	Present	Present	1.82
5	ST1404	10	15	T	++++	Present	Present	1.62
6	ST1413	11	50	A, Cm	++++	Present	Present	1.49
7	ST1425	12	20	A, Cm, T, C	++++	Present	Present	0.98
8	ST1430	10	14	A, Cm, T, C	+++	Present	Present	1.51
9	NIB38	15	30	A, Cm, T, C	++++	Present	Present	1.58
10	ST1004	10	15	A, Cm, T, C	+++	Present	Present	1.42
11	1577	15	30	T	++++	Present	Present	1.49
12	1670	15	45	Cm	++++	Present	Present	1.08
Mean ± SD		13.125 ± 1.96	32.25 ± 12.78					1.47 ± 0.23
Min–max		10–15	15–50					0.98–1.82
13	1671-S	10	32	Cm	++	Present	Present	1.38
14	1890-XP	7	20	-	++	Present	Present	1.42
15	1350-XZ	15	15	T	++	Present	Present	1.39
16	1420	10	20	A	++	Absent	Present	0.99
17	H56	7	31	A, Cm, T, C	++	Present	Present	1.10
Mean ± SD		9.8 ± 3.27	23.6 ± 7.5					1.25 ± 0.197
Min–max		7–15	15–32					0.99 –1.42
18	1421	10	17	-	+	Present	Present	1.32
19	1422	9	18	T	+	Present	Present	0.96
20	1429	11	15	-	+	Present	Present	0.90
21	1876	8	20	Cm	+	Present	Present	1.03
22	1987	9	17	A	+	Absent	Present	1.00
23	2534	11	14	-	+	Absent	Present	0.99
Mean ± SD		8.88 ± 1.21	16.51 ± 2.13					1.03 ± 0.14
Min–max		8–11	14–20					0.90 –1.32
24	NIB25	6	12	-	–	Absent	Absent	
25	1680-S	5	10	-	–	Present	Present	
26	1681-S	10	16	-	–	Absent	Absent	
27	1423	6	17	-	–	Present	Present	
28	1424	8	13	Cm	–	Present	Present	
29	1428	7	15	A	–	Absent	Absent	
30	1431	6	10	T	–	Absent	Absent	
Mean ± SD		6.85 ± 1.67	13.28 ± 2.81					0.87 ± 0.076
Min–max		5–10	10–17					0.76–0.99

PCR, polymerase chain reaction; LDH, lactate dehydrogenase; SD, standard deviation.
[a] Days were counted from the day the disease was diagnosed.
[b] Cells grown in LB broth/cells from matrix/planktonic phase showed the same pattern: chloramphenicol (Cm), ampicillin (A), trimethoprim (T), ciprofloxacin (C).

The presence of the Vi antigen is also known to increase the infectivity of *S*. Typhi and the severity of disease in volunteers.[27,28] Like biofilm, the Vi capsule, being exopolysaccharide in nature, may have a significant role in biofilm formation and persistence of infection. But as our data suggest, the *via*B operon is found in both biofilm and non-biofilm producers and thus is not a significant contributor to biofilm production.

The type IV B pilus of the enteropathogenic bacteria *S*. Typhi is a major adhesion factor during entry of this pathogen into gastrointestinal epithelial cells.[29] In this study, detection of type IV B pili in all biofilm producers strongly suggests its preliminary role in biofilm production. Unfortunately animal models are not successful for *S*. Typhi, which is a strict human pathogen, and in vivo studies are difficult and often inconclusive. Therefore, considering the difficulties regarding in vivo studies to show the prolonged carrier state, our findings provide valuable information in this regard.

In conclusion, it was found that the time to clearance of *S*. Typhi from typhoid patients after recovery (as gauged by PCR on stool samples) is directly related to biofilm production capability. The period between blood and stool PCR negativity differs from patient to patient and may extend up to 2 months. The presence of biofilm does not alter the drug resistance profile of the bacteria, but provides physical protection which results in delayed clearance probably due to 'persisters'. It was also found that the presence of Vi capsule has no relevance to biofilm production, but that type IV B pili have a significant effect.

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Ethical considerations: The study was reviewed and approved by the review boards of the participating institutes. Informed consent was provided by all participants or their parents.

Conflict of interest: No conflict of interest to declare.

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