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# BIOFILM: AN IMPORTANT FACTOR IN THE PREVALENCE OF MDR *SALMONELLA TYPHI*

**CHAUHAN N, KHAN MA AND FAROOQ U***

Molecular and Immuno-parasitology research laboratory, Faculty of applied sciences and biotechnology, Shoolini University of Biotechnology and Management Sciences, Bajhol, Solan, 173212, Himachal Pradesh, India

*Faculty of Dentistry, Taif University, Taif, KSA

***Corresponding Author: E Mail: ufarooq8@gmail.com**

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## ABSTRACT

Typhoid has become an endemic disease in developing countries including India. *Salmonella typhi,* the major causative agent of human typhoid has the ability of biofilm formation. Hence, it is associated with the biomaterial infections. The biofilm formed by these bacteria is the major contributor to its resistance and persistence in the host. The bacteria inside the biofilm are well protected against various environmental stresses, antimicrobials, disinfectants, and the host immune system. Due to these characteristics it seems extremely difficult to eradicate the bacteria from the host. In this study, *in vitro* biofilm formation by MDR isolates were analyzed using Adhesion assay, Tube-method and Microtitre plate assay. A total of 40 clinical isolates were collected from confirmed *S. typhi* infected patients from Solan, district of Himachal Pradesh and were studied for cultural characteristics, biochemical identification and for the resistant pattern. It was observed that out of 40 clinical isolates, 14/40 isolates were MDR for several classes (Penicillins, Sulfonamides, Macrolids, Glycopeptides, Tetracycline and Antimycobacterials) of antibiotics. Among these 14 MDR isolates, 10 were found biofilm forming by the test tube method and 12 were on the surface of microtitre plate. Therefore, Microtitre plate method can be used upon the tube method to assess the *S. typhi* virulence factor associated with the formation of biofilm and for examining the efficacy of biofilm destruction modalities.

**Keywords: *Salmonella typhi*; Biofilm production; Multi drug resistant; tube method; microtitre plate assay**

## INTRODUCTION

The incidence of typhoid is increasing in continuous manners and the emergence of multidrug resistant *Salmonella typhi* has become an alarming feature. The ability of Biofilm- formation has been reported in *Salmonella enterica* serovar Typhi (*S. typhi*) [1]. *S.typhi* is the etiological agent of typhoid fever with global incidence of 21.7 million and 217,000 deaths annually [2]. Several case studies showed that the major risk for enteric fever implies with contaminated water and food as important transmission routes [3, 4, 5, 6]. Typhoid is a communicable disease for as long as the infected person excretes bacteria in stool. These bacteria usually not diagnosed from the stool samples after a week of illness when symptoms get resolved. Although, a percentage of this infection results in asymptomatic carriage of salmonellae, due to the formation of Biofilms that contributes in the development of the carrier state in patients [7].

Most of the patients after recovery from the infection are able to eliminate the bacterium completely from their bodies without complications. However, an approximately 5-10% of infected individuals may remain as carriers and continuously shed *S. typhi* in their stools [8]. A recent study showed that *S. typhi* is frequently associated with the gallstones presence in asymptomatic human carriers, in which the pathogen colonies persists as Biofilm on the gallstones [1]. Despite the caustic nature of bile in gallbladder, Biofilms allow the continual shedding and reattachment of individual cells, contributing to the spread of bacteria via urine and faeces, particularly in the human host [9, 10].

A biofilm is a bacterial colony adherence to solid surface that secretes a self-initiated, protective exopolysaccharide matrix [11, 12]. Biofilms are defined as structured communities of bacterial cells enclosed in a self produced polymeric matrix adherent to inert or living surfaces [13, 14, 15]. The ability to form biofilm through the complex interaction of bacteria is important for bacterial survival within the human host. Moreover, well-established biofilm makes it difficult for both the innate and adaptive immune responses of the human hosts to eliminate the pathogen [12, 17]. Bacterial biofilms are the predominant mode of bacterial growth, reflected in the observation that approximately 80% of all bacterial infections are related to biofilms [18, 19]. Bacteria in biofilms are well protected against environmental stresses, antimicrobial

effects [20], disinfectants, and the host immune system [12], and as a consequence are extremely difficult to eradicate [11]. 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 microplate are up to 2000-fold more resistant to ciprofloxacin as compared to planktonic cells [12]. This is particularly concerning, as ciprofloxacin is commonly used to treat *Salmonella* infections [20].

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 reported that *S. typhi* can also cause disease in the absence of capsule [21, 6]. As biofilm has a protective role similar to capsule, this can be hypothesized that its presence may have a shielding role and be a basis for longer survival in the body, thus substantiating the carrier status and resistance. This study was designed to evaluate the role of biofilm produced by *S. typhi* in MDR emergence.

## MATERIAL AND METHODS

### Ethical Justification

The project has been approved by institutional ethical committee of Shoolini University, Solan, HP, India, wide Letter Ref. Number: SUBMS/IEC/15/13.

### Bacterial Isolates

Total of 40 (cultural, WIDAL and ELISA confirmed) clinical isolates was selected from infected inpatients from Solan district of Himachal Pradesh. The bacterial strains were characterized and identified on the basis of colonial morphology studies on differential and specific media and confirmed by biochemical characterization. The cultures were inoculated on trypticase soy agar (TSA), 16% (vol/vol) glycerol and kept at -20°C. Standard strains of *Salmonella typhi* MTCC 733 was used as reference strain. Detection of biofilm was achieved by Microtitre dish assay and Tube Method.

### Microtitre dish assay

An overnight culture of *S. typhi* on TSA (Tryptic Soy Agar) was diluted 200-fold with culture medium (TSB+1% glucose), of which 200µl was added to the wells of a 96-well polystyrene microtitre plate and Incubated at 37°C for 24 h. After the incubation period wells were aspirated carefully and washed 3-4 times with PBS (pH 7.2). Cells were emptied, fixed and quantified for biomass production. The cell density was determined at 490nm with a microtitre plate reader (BioTek ELx800). Biofilm formation in each well was analyzed by 0.1 safranin/crystal

violet staining methods [8]. Each experiment was performed at least three times and in each experiment the samples were prepared in six wells.

**Adhesion assay**

An overnight culture was diluted 200-fold with 10 ml TSBg (Tryptic Soy broth and glucose) and then placed in a 9-cm Petri dish. After incubation at 37°C for 24h, the plate was tilted by placing it on the edge of a Petri plate cover. It was observed that if biofilm was formed, the top portion of the plate remained clear or if present cells were observed as clumps in the medium of lower half of the plate.

**Tube method**

*S. typhi* isolates were tested for biofilm production by a modification of the standard method of Christensen *et al*. [19]. Two milliliters of trypticase-soy broth in 12 x 75mm borosilicate test tubes were inoculated with loopful overnight bacterial suspension and incubated for 48hours at 37°C. After that the contents were decanted and washed with PBS (pH 7.3±0.2) and left to dry at room temperature. Afterwards, the tubes were stained with 4% solution of safranin. Each tube was then gently rotated to ensure uniform staining and the contents were gently decanted. The tubes were placed upside down to drain and then observed for biofilm formation. The strains were considered positive for biofilm formation after observing a visible film lined the wall and bottom of the tubes. Ring formation at the liquid interface was not regarded as indicative of biofilm formation. The results were scored visually as 0-absent, 1-weak, 2-moderate, 3-strong [11].

**RESULTS**

The present study was conducted on 40 clinical strains of *S. typhi* which were isolated from blood specimens of confirmed typhoid patients. Out of 40 clinical isolates of *S. typhi*; 14/40 (35%) isolates were considered multi drug resistant for several classes of antibiotics. Out of 14 MDR strains 71.43% (10/14) found Biofilm positive by tube method and 85.71% (12/14) by microtitre plate assay, while a very few strains were found positive by adhesion assay of petriplates. The qualitative tube adherence test was visually assessed for the degree of adherence of *S. typhi* to the sides of borosilicate test tubes. The interpretation of this test was recorded as strong adherence 3 (+++), moderate 2 (++), weak adherence 1 (+) or negative 0 (-) (Figure 1, Table-1).

The quantitative microtitre plate adherence assay was primarily assessed by visual identification of the degree of adherence of *S.typhi* to the bottom of each well (Figure-2,

Table-1) and further by quantifying with microtitre reader. The cut off value was calculated by taking average of negative control and by addition of 3 times of SD. The adhesion of bacterial biofilm was assessed in the following manners: $OD>4xOD_C$ were considered as strongly adherent (+++); $4xOD_C \geq OD > 2xOD_C$ was considered moderately adherent (++), $2xOD_C \geq OD > OD_C$ were considered Weakley adherent (+) and those having $OD_C \geq OD$ were considered as negatively adherent (-) whereas $OD_C$ was the cut off optical density.

It was clearly observed that 4/14 (28.57%) strains were strong biofilm producing, 5/14 (35.71%) were moderate and 3/14 (21.43%) were weak biofilm producers. Whereas 2/14 (14.28%) strains do not form biofilm, the reason behind these observations may be that biofilm was not responsible for the multidrug resistance in these strains or there may be other factors coding for their resistance.

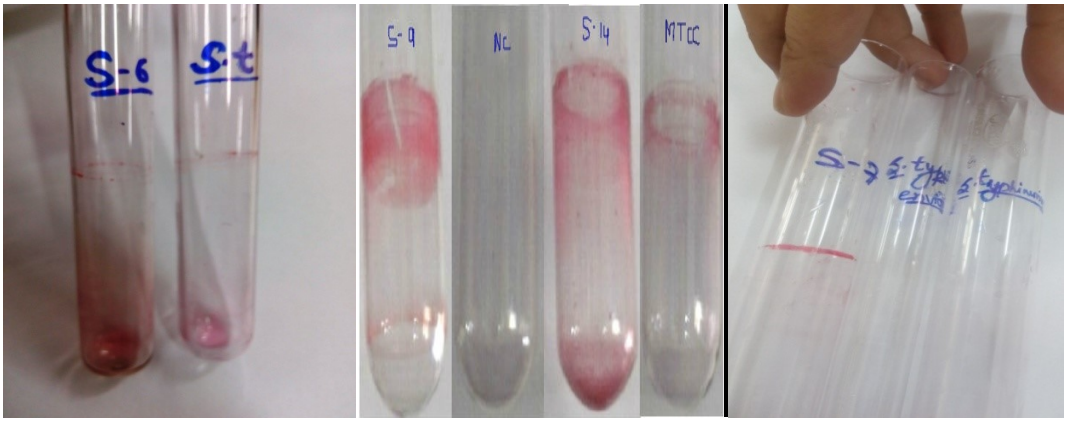


**Figure-1: Tube test showing high, moderate and weak Biofilm production in various strains of *S. typhi* as compared to positive (MTCC) and negative controls.**

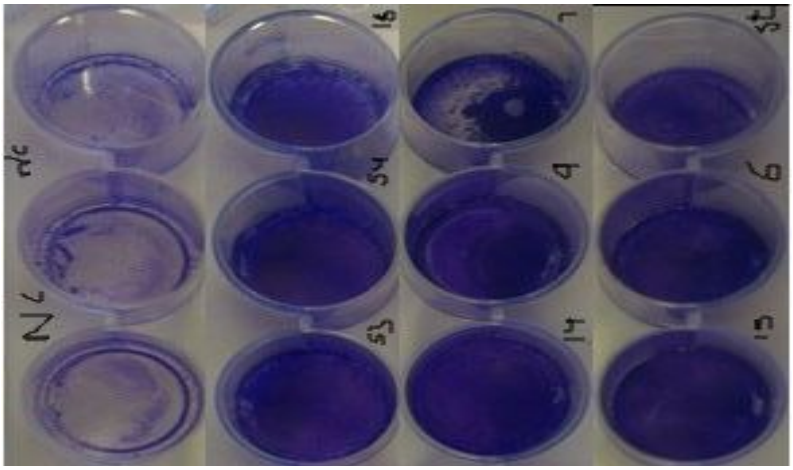

**Figure-2: Showing the presence of Biofilm formation by MDR *S. typhi* in flat-bottom microtitre plate as compared to standard and non-MDR strains (NC)**

**Table 1: Showing Biofilm formation among MDR isolates of *S. typhi* in comparison to positive and negative controls**

| MDR isolates | Tube Assay | Adherent Assay |
|---|---|---|
| ***S. typhimurium*** | **0** | - |
| ***S. typhi (NC)*** | **0** | - |
| **ST (MTCC)** | **1** | ++ |
| **S-3** | **1** | + |
| **S-4** | **0** | + |
| **S-5** | **0** | - |
| **S-6** | **3** | +++ |
| **S-7** | **3** | +++ |
| **S-9** | **3** | +++ |
| **S-10** | **2** | ++ |
| **S-11** | **0** | - |
| **S-12** | **2** | ++ |
| **S-13** | **0** | - |
| **S-14** | **3** | +++ |
| **S-15** | **2** | ++ |
| **S-16** | **2** | ++ |
| **S-17** | **0** | + |

**Score-3, 3> OD> 2 (>3.32) = strongly adherent (+++); Score-2, 2> OD> 1 (1.16-3.32) = moderate adherent (++); Score-1, 1> OD > 0.5 (0.58-1.16) = Weakley adherent (+); Score-0, $OD_C$=OD < 0.5 (0.58) = negatively adherent (-) [11].**

## DISCUSSION

Typhoid is a communicable disease for as long as the infected person excretes *S. typhi* in the feces. As per the availability of major treatment and prevention efforts, millions of new typhoid cases evolve worldwide every year. The bacterium colonize systematically, mostly in the gall bladder, and remains there long after symptoms subside to be a reservoir for the further spread of the disease [22]. The excretion of the bacteria in stool begins about a week after the onset of illness and continues throughout the convalescence period and remains for a variable period thereafter [23]. Biofilm formation played a very significant role in establishing long-term colonization of bacterial cells that continuously shed for extended periods [24]. In the present study, we tried to find a correlation between this MDR and the Biofilm production capability of isolates, if any. We found that MDR isolates were of high-grade biofilm producers. It was reported earlier that the maximum period was observed 50 days for bacterial shedding (average 32.25 days) in post-infection case of

high biofilm producers, and was not more than 17 days (average 13.28 days) in the case of non-biofilm producers. Thus the presence of biofilm in *S. typhi* may be related to the length of the carrier state in a patient after recovery. Biofilm formation may prolong the carrier state, but remains to be evaluated whether this was due to the physical protective effect or the biofilm forming MDR strains. These studies will need to be exposed in comparison to the planktonic phase bacteria. In recent studies, 194 strains of *S. enterica,* isolated from infected children were investigated for their ability to form Biofilms on silicone disks in comparison to planktonic cells which were susceptible to nine antimicrobial agents. The findings indicate that 56% of these strains were able to form biofilms [25]. The biofilm forming strains showed increased antimicrobial resistance to several classes of antibiotics as compared to the planktonic bacterial strains (non biofilm producers). Once the antibiotic level drops, the persisters may multiply, explaining the relapsing nature of biofilm infections. The presence of the Vi antigen is also known to increase the infectivity of *S. typhi* and the severity of disease in volunteers [26, 27]. Similar to biofilm, the Vi capsule antigen is an exopolysaccharide and play a significant role in biofilm formation and persistence of infection. The type IV B pilus of the entero-pathogenic bacteria *S. typhi* is a major adhesion factor during entry of this pathogen into gastrointestinal epithelial cells [22]. Unfortunately, animal models are not much successful in the case of *S. typhi,* because it is a strict human pathogen. Hence, *in vivo* studies are difficult and often inconclusive. Therefore, considering the difficulties regarding *in vivo* studies, it is more difficult to study the prolonged carrier state. Our findings provide valuable information in the regards of correlation between the formation of biofilm and the prevalence of MDR strains.

**CONCLUSION**

It was concluded from the present study that microtitre dish assay is the most reliable method over the Petri dish and test tube adherence assays for the detection of Biofilm in MDR isolates of *S. typhi.* Biofilm may be the main cause of spreading of MDR strains of *S. typhi* and increasing the incidence of this disease day by day. Also the biofilm cause a multitude of problems in the medical field, like adhesions in the catheters or several diagnostic tools. A detailed study was needed upto the molecular level to find the genes involved in the formation and invasion of biofilm and the spread of MDR strains.

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