International Food Research Journal 18(4): 1493-1498 (2011)

Optimization of microtitre plate assay for the testing of biofilm formation ability in different *Salmonella* serotypes

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Abstract: *Salmonella* has the ability to form biofilms on food-processing surfaces including plastic, potentially leading to food product contamination. The objective of this study was to determine the biofilm ability of different *Salmonella* serotypes for which microtiter plate assay was optimized. The assay was optimized with growth of *Salmonella* cells in Luria-Bertani broth for 48 h incubation, fixing of cells by heat (80^0C, 30 min) and staining with 0.5% crystal violet stain and measuring crystal violet absorbance, using solubilizing (ethanol/acetone, 80: 20%) solution. A total of 151 strains of *Salmonella* consisting reference and field isolates from diverse sources belonging to 69 serotypes were screened for biofilm production. Majority of strains (87, 57.61%) were found to be moderate biofilm producers, while 34 (22.52%) and 29 (19.21%) strains were weak and strong biofilm producers, respectively. One strain belonging to *S.* Munster serotype did not produce any biofilm. The optimized microtitre plate assay can be effectively used for the assessment of biofilm ability of *Salmonella* strains. The highlight of the study was the testing of large number of *Salmonella* strains belonging to several serotypes. Majority of *Salmonella* strains are shown to form biofilms on plastic surface which has significance for food industry

Keywords: Biofilm, food industry, microtitre assay, *Salmonella*

Introduction

Salmonella is one of the most important foodborne pathogens, responsible for food borne illness associated with variety of food products. The organism has the capability to adhere and form biofilms on surfaces such as plastic, glass, stainless steel or rubber surfaces (Hood and Zottola, 1997; Wong, 1998; Sommer *et al.,* 1999, Sinde and Carballo, 2000; Joseph *et al.,* 2001). The biofilms, when formed on these contact surfaces, could be the continuous source of contamination and lead to serious implications in industrial, environmental, public health and medical situations (Hall-Stoodley *et al.*, 2004). Common sites for the presence of *Salmonella* spp. in food processing plants are filling or packaging equipments, floor drains, walls, cooling pipes, conveyors, collators for assembling product for packaging, racks for transporting products, hand tools or gloves, freezers, etc, which are usually made of plastics (Pompermayer and Gaylarde, 2000). Therefore, assessment of biofilm formation capabilities of *Salmonella* is important.

Several methods have been developed for the cultivation and quantification of biofilms (Deighton *et al*., 2001; Arciola *et al*., 2002; Harraghy *et al*., 2006), but no standardized protocol for assessment of biofilm formation by different bacterial species has been established so far. However, the microtiter plate method remains among the most frequently used assays for investigation of biofilm and a number of versions have been used for the *in vitro* bacterial biofilm formation, which makes it difficult to compare the results and find an acceptable method. Microtitre plate being made up of plastic has the added advantage, as biofilm formation on it will have direct relation to plastic used in food industry. Djordjenvic *et al*. (2002) compared the biofilm formation ability by microtiter plate assay and quantitative epifluorescence microscopy and reported that former assay revealed greater differences in biofilm production than did the microscopy biofilm assay. They also opined that microtiter plate assay can be used as rapid and simple method to screen difference in biofilm formation between strains. An and Friedman (2000) also reported microtiter plate procedure as an indirect method for estimation of bacteria *in situ* and can be modified for various biofilm formation assay.

Biofilm formation by *Salmonella* spp. has been exhaustively studied on plastic surfaces (Romling and Rohde, 1999; Joseph *et al*., 2001; Djordjevic *et al*., 2002; Stepanovic *et al*., 2003; Pui *et al*., 2011), but many of these studies were carried out with a limited number of strains. The objective of the present work was to study the influence of various factors (growth medium, incubation period, fixation of adhered cells

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and staining) in development of biofilm by *Salmonella* on microtiter plate. The study was also conducted to compare the ability of different *Salmonella* serovars to produce biofilm on plastic surface.

Materials and methods

Bacterial strains

A total of 151 *Salmonella* strains belonging to 69 different serotypes (Table 1) maintained at National *Salmonella* Centre, Indian Veterinary Research Institute, Izatnagar were assessed in the present study. These strains included both reference (80) and field strains of different host origin (71). All the strains were tested for their purity, morphological and biochemical characteristics and maintained by periodical sub culturing in nutrient agar slants (Himedia Laboratories, Mumbai).

Table 1. Biofilm ability of different *Salmonella* strains in microtiter plate assay

S.No	Serotype	No. of strains	Biofilm		
			Weak	Moderate	Strong
1	*S.* Typhimurium	31	3 (9.68)	22 (70.97)	6 (19.35)
2	*S.* Gallinarum	14	-	10 (71.43)	4 (28.57)
3	*S.* Enteritidis	12	-	8 (66.67)	4 (33.33)
4	*S.* Anatum	11	1 (9.09)	8 (72.73)	2 (18.18)
5	*S.* Dublin	7	1 (14.29)	4 (57.14)	2 (28.57)
6	*S.* Cholerasuis	5	1 (20.0)	4 (80.0)	-
7	*S.* Paratyphi B	3	1 (33.33)	2 (66.67)	-
8	*S.* Paratyphi A	1	-	1 (100)	-
9	*S.* Berta	3	1 (33.33)	2 (66.67)	-
10	*S.* Abortus equi	3	-	3 (100)	-
11	*S.* Derby	2	2 (100)	-	-
12	*S.* Montevideo	2	-	2 (100)	-
13	*S.*Agona	1	-	1 (100)	-
14	*S.* Milwaukee	1	1 (100)	-	-
15	*S.* Panama	1	1 (100)	-	-
16	*S.* Rubislaw	1	-	1 (100)	-
17	*S.* Sandiago	1	-	1 (100)	-
18	*S.* California	1	1 (100)	-	-
19	*S.* Weslaco	1	1 (100)	-	-
20	*S.* Schleissleim	1	-	1 (100)	-
21	*S.* Duesseldrof	1	1 (100)	-	-
22	*S.* Bredeney	1	-	-	1 (100)
23	*S.* Rostock	1	1 (100)	-	-
24	*S.* Newington	1	-	1 (100)	-
25	*S.* Oranienberg	1	1 (100)	-	-
26	*S.* 4,5,12:eh:-	1	1 (100)	-	-
27	*S.* 6,7:y:-	1	-	1 (100)	-
28	*S.* Tennessee	1	-	1 (100)	-
29	*S.* Worthington	1	1 (100)	-	-
30	*S.* Virginia	1	-	1 (100)	-
31	*S.* 3,10:-:1,6	1	-	-	1 (100)
32	*S.* Seftenberg	1	-	1 (100)	-
33	*S.* Reading	1	1 (100)	-	-
34	*S.* Pullorum	1	-	1 (100)	-
35	*S.* Alachua	1	-	-	1 (100)
36	*S.* Weltevreden	1	1 (100)	-	-
37	*S.* Bredeney	1	-	1 (100)	-
38	*S.* Adelaide	1	-	1 (100)	-
39	*S.* Brancaster	1	-	1 (100)	-
40	*S.* Bareilly	1	-	1 (100)	-
41	*S.* Heidelberg	1	-	1 (100)	-
42	*S.* Artis II	1	-	-	1 (100)
43	*S.* Bergen	1	-	1 (100)	-
44	*S.* Bredeny phI	1	1 (100)	-	-
45	*S.* Cerro	1	1 (100)	-	-
46	*S.* Budapest	1	-	-	1 (100)
47	*S.* Bonariensis	1	-	-	1 (100)
48	*S.* Dusseldrof	1	1 (100)	-	-
49	*S.* Dahlem	1	1 (100)	-	-
50	*S.* Deversoir	1	1 (100)	-	-
51	*S.* Chester	1	-	-	1 (100)
52	*S.* Typhi 901	1	-	1 (100)	-
53	*S.* Humber II	1	-	-	1 (100)
54	*S.* Greenside	1	1 (100)	-	-
55	*S.* Essen	1	1 (100)	-	-
56	*S.* Java	1	1 (100)	-	-
57	*S.* Grumpensis	1	1 (100)	-	-
58	*S.* Javiana	1	-	1 (100)	-
59	*S.* Huittingfors	1	-	1 (100)	-
60	*S.* Gaminara	1	1 (100)	-	-
61	*S.* Glostrup	1	1 (100)	-	-
62	*S.* Haarlem	1	-	1 (100)	-
63	*S.* Munster	1	NP		
64	*S.* Illinois	1	-	1 (100)	-
65	*S.* Moscow	1	-	-	1 (100)
66	*S.* Kentucky	1	-	-	1 (100)
67	*S.* Locarno	1	1 (100)	-	-
68	*S.* Niarembe	1	1 (100)	-	-
69	*S.* Newport	1	-	-	1 (100)
Total		**151**	**34 (22.52)**	**87 (57.61)**	**29 (19.21)**

Inoculum preparation

All the strains of *Salmonella* spp. were sub cultured into Luria-Bertani broth (LB, Difco, USA) individually and incubated aerobically at 37^0C for 24 h. The bacterial growth was harvested by centrifugation of culture, pelleted cells was dissolved in normal saline solution. The bacterial growth was quantified as per standard Nephelometric tube method and the density of bacterial suspension was adjusted so as to give counts of approximately 10^9 cells/ml.

Microtiter plate assay

Media and incubation

Initially biofilm formation of *Salmonella* was investigated in two different broths i.e TSB (Tryptic-Soy broth) and LB (Luria-Bertani) to compare the effect of nutrients availability in biofilm promotion at 37^0C for different time intervals of 24, 48 and 72 h as it considerably influences the amount of biofilm produced and highest density of cells in biofilm reach at optimum incubation period.

Fixation

Two protocols were compared for fixation of cells in the plates. In first trial, cells were fixed with methanol, and in another, heat fixation at 80^0C was employed.

Staining

Two different concentrations of crystal violet stain (0.1% and 0.5%) were compared.

Screening of Salmonella *strains for biofilm formation*

Based on the preliminary trails, the following protocol was used to screen 151 strains of *Salmonella*. From each individual culture, 20 µl samples of in exponential phase and 180 µl of LB broth were dispensed in the wells of sterile 96-well flat-bottomed microtiter plate (Nunc) and kept for incubation at 37^0C for 48 h. Each strain was inoculated into at least 8 wells. The control well contained only LB broth without inoculation. After incubation, unbound cells were removed by inversion of microtiter plate, followed by vigorous tapping on absorbent paper. Subsequently, adhered cells were fixed for 30 min at 80^0C.

Adhered cells were stained by addition of 220 µl of crystal violet (0.5%) for 1 min. The stain was removed by exhaustive washing with distilled water. The plates were then allowed to dry. In order to quantify adhered cells, 220 µl of decolouring solution (ethanol/acetone, 80:20%) was added to each well for 15 min. The absorption of the eluted stain was

measured at 590 nm. The strains were classified into the three categories: weak, moderate and strong biofilm producers as per Stepanovic *et al*. (2004).

Statistical analysis

Data obtained from above experiments was subjected to statistical analysis as per standard procedure of Snedecor and Cochran (1989).

Results

Preliminary studies to optimize the microtiter plate assay for biofilm formation was carried out using nine different serotypes of *Salmonella* (*S.* Abortus equi, *S*. Paratyphi, *S.* Budapest, *S.* Typhimurium, *S*. Grumpensis, *S.* Typhi 901, *S*. Berta, *S.* Java and *S.* Bonariensis).

Growth medium

Biofilm production in LB broth was much greater than that observed in TSB. In LB most of the serotypes were found to be strong and moderate biofilm producers, while in TSB they produced weak biofilm. For example, the average OD for *S.* Typhi 901 was 1.51±0.4 (OD ± SE) in LB broth at 48 h, but in TSB its OD was only 0.65 ±0.3, just half of the value observed for LB. The same patterns of differences were also observed among other serotypes of *Salmonella* in both the growth medium (Figures 1 and 2). Therefore, LB broth was selected as the medium for biofilm formation in this study.

Incubation period

At 24 h of contact time none of the *Salmonella* strains formed biofilms and the average OD ranged from 0.36 ± .02 to 0.74 ± .04, which was equal to the control OD (i.e. 0.43 ±.03). Later on, adhesion of cells to microtiter plate increased linearly. After 48 h all strains were found to produce biofilms. The average OD for S. Java was 0.74 ±.04, 1.22 ±.02 and 0.70±.04 at 24 h, 48 h and 72 h, respectively. The same pattern was also observed for other strains (Figures 1 and 2). Optimum results were observed after 48 h of incubation and were considered sufficient to evaluate the ability of *Salmonella* serotypes to produce biofilm.

Fixation

Comparative fixation of adhered cells on microtiter plate by methanol and by application of heat (80^{0}C) revealed that on fixing the cells with methanol (250 µl for 30 min), most strains were found to be either non- biofilm producers or weak biofilm producers (average OD ranging from 0.15 ±.01 to 0.58 ±.09).

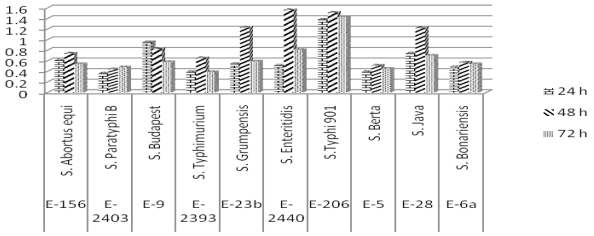

Figure 1. Effect of incubation time on biofilm formation in LB broth

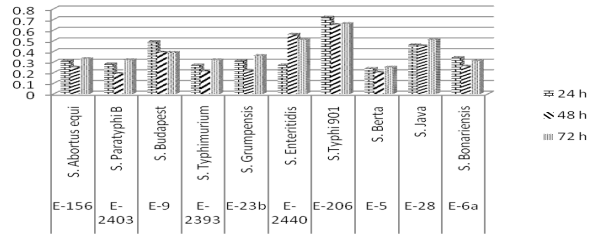

Figure 2. Effect of incubation time on biofilm formation in TSB

In contrast, fixing of cells at 80^{0}C for 30 min reveled most strains to be strong or moderate biofilm (1.17 ±.07 to 2.85±.09) producers (Figure 3).

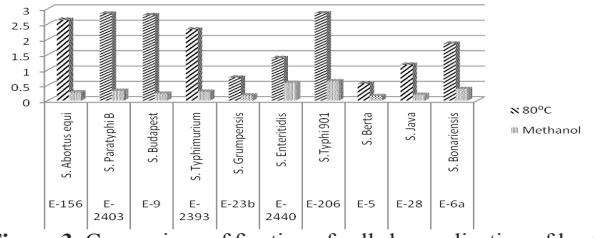

Figure 3. Comparison of fixation of cells by application of heat and methanol after 48 h

Staining

Staining with 0.5% crystal violet revealed better results in comparison to staining with 0.1% crystal violet for 1 min.

Screening of biofilm formation in Salmonella *strains*

From the preliminary trials, the optimum condition/procedure for the assay was LB broth as growth medium, incubation at 48 h, fixation at 80^{0}C and staining with 0.5% crystal violet stain was considered optimum for judging the efficacy of *Salmonella* to form biofilms.

A total of 151 strains of *Salmonella* belonging to 69 serotypes were screened for biofilm production. Majority of strains (87, 57.61%) were found to be moderate biofilm producers, while 34 (22.52%) and 29 (19.21%) strains were weak and strong biofilm producers, respectively. Only *S.* Munster serotype did not produce any biofilm (Table 1).

Variable results were observed for different strains belonging to same serotype. Among 31 *S.* Typhimurium strains, 22 (70.97%) produced moderate biofilms, followed by 19.35% strong and 9.68% weak biofilm producers. Similarly, among 14 strains of *S.* Gallinarum 11 (71.43%) were strong,

while 4 (28.57%) were moderate biofilm producers. Likewise, variable results were observed for different strains belonging to same serotype.

Discussion

The process of biofilm formation by microorganisms is influenced by various factors including nutrients level, pH, temperature, incubation period, ionic strength, culture concentration, etc., but the bacterial cell surface appendages (fimbriae, flagella, curli, exopolysaccharides, outer membrane proteins) and the contact surface characterstics are the most important among all of them as its formation begins when bacterial cells encounters a suitable surface and its outer surface adheres to the substratum.

Interactions between bacterial cells and inorganic surfaces are different for adhesion onto hydrophobic or hydrophilic surfaces (Sommer *et al.*, 1999). Among the different surfaces (Granite, stainless steel, glass, rubber, mica, plastic) used by various investigators for *Salmonella* biofilm, plastic surfaces are considered efficient in comparison of others materials (Sinde and Carballo, 2000; Donlan, 2002). The suitability of plastic surfaces for bacterial attachment is due to its hydrophobic nonpolar nature with little or no surface charge, while other materials like glass, stainless steel, mica are hydrophilic and negatively charged (Sinde and Carballo, 2000; Djordjevic *et al*., 2002). In this study, we used plastic surface for biofilm formation and found it be efficient.

Composition of media is also considered as important factor which affects the ability of *Salmonella* to produce biofilm (Hood and Zottola, 1997; Stepanovic *et al.*, 2004). In this study, TSB and LB were selected for the comparison. TSB is considered as less rich media and most frequently used for biofilm formation for various bacterial spp. including *Salmonella,* as it has been suggested that higher number of cell adhesion takes place when bacteria are grown in low-nutrient media (Prakash and Krishnappa, 2003). Gerstel and Romling (2001) also reported that when the levels of nutrients are less or bacterial cells were in stationary phase, maximum expression of aggregative fimbriae took place, which played role in biofilm formation. On the contrary, LB (nutrient rich) was found to be more effective in promoting biofilm formation in our study. Other investigators also reported that *Salmonella* spp. needs rich nutrients for biofilm formation (Hood and Zottola, 1997; Stepanovic *et al*., 2004). However, various media like BHI, meat broth, reconstituted skim milk, diluted meat juice, TSB with additional glucose etc. were in use as it has been reported that biofilm formation by different bacterial spp. is independent of the concentration of medium (Hood and Zottola, 1997).

The duration of incubation period also considerably influences the amount of biofilm produced as biofilm density increases with the incubation period. We compared 24 h, 48 h and 72 h of incubation period. No biofilm formation was observed at 24 h. In contrast great majority of biofilm studies recommended the incubation for 24 h (Deighton *et al*., 2001; Moretro *et al*., 2003; Mathur *et al*., 2006; Vasudevan *et al*., 2003; Djordjevic *et al*., 2002; Stepanovic *et al*., 2004). The above reported studies were carried out for Gram positive bacteria, which may explain the variation in results. In our study, 48 h incubation was found to be most efficient in bioflm formation by *Salmonella* spp. The results of our study are in agreement with other studies, where 48 h of incubation period was suggested for biofilm formation in *Salmonella* spp. (Brendan and Ethan, 2005; Joseph *et al*., 2001). At 72 h PI decrease in OD was observed, which indicated that the saturation level was already reached. Some researchers also used prolonged incubation of 96 h to 10 days for biofilm formation (Hood and Zottola, 1997; Prakash and Krishnappa, 2002; Malcovaa *et al*., 2008).

In earlier studies Bouin's reagent was used as an effective fixative for biofilm, but later on it was replaced by the alternatives like formaldehyde, methanol, etc., as it contains explosive chemicals (Baldassarri *et al*., 1993) and also increases background staining and blackening of biofilm and reduced the average OD up to 20% (Wilcox, 1994). Genevaux *et al*. (1996) used temperature of 80^0C for fixation of adhered cells. The fixation of attached cells by exposing them at 80^0C for 30 min was found to be comparatively better than fixation by methanol in our study. Our results are in agreement with the Baldassarri *et al*. (1993) who also reported that the heat fixation appears to be the method of choice for cells fixation in biofilm formation studies.

Crystal violet is most widely used for the staining of biofilm cells and its different concentrations (1%, 2%) were used by various workers (Djordjevic *et al*., 2002; Wilcox, 1994). Two different concentrations of crystal violet stain (0.1% and 0.5%) were compared for staining of adhered fixed cells. Staining with 0.5% revealed better results in comparison to staining with 0.1% crystal violet for 1 min. The results are corroborative to the finding of Stepanovic *et al*. (2003).

Evaluation of biofilm formation ability by *Salmonella* in this study revealed that these bacteria

possess capacity for biofilm formation on plastic surfaces. Our results are in agreement with other studies which showed that *Salmonella* are able to form biofilm on plastic surfaces (Joseph *et al.*, 2001; Djordjevic *et al.,* 2002; Stepanovic *et al.,* 2003; Stepanovic *et al*., 2004). Of the 151 strains of Salmonella tested, 56.61% were found to be moderate biofilm producers, while 22.59% and 19.21% strains were strong and weak biofilm producers, respectively. Only 1 strain did not produce any biofilm. Stepanovic *et al*. (2004) found 72.9% of the 121 *Salmonella* strains to be biofilm producers of which, 66.3% were strong biofilm producers. In another study, employing pellicle formation at liquid-air interface method in LB broth, observed only 75% of 204 isolates to be biofilm producers (Solano *et al.*, 2002). Different extent of biofilm formation by *Salmonella* was observed among different serotypes. Also, the source of *Salmonella* isolates does not seem to affect the ability to form biofilm on plastic surfaces (Stepanovic *et al.*, 2004).

The optimized microtitre plate assay can be effectively used for the assessment of biofilm ability of *Salmonella* strains. Ability of majority of *Salmonella* strains to form biofilms on plastic surface as also shown in this study is of significance for food industry.

Acknowledgment

Authors are thankful to the Director, Indian Veterinary Research Institute, Izatnagar for providing necessary facilities to carry out the work. Financial support received from Department of Biotechnology, Ministry of Science and Technology, Govt. of India, New Delhi for this study is also gratefully acknowledged.

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