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Shamanth Rakkith RN
M.V.Sc., Department of Veterinary Public Health and Epidemiology Veterinary College, Shivamogga, Karnataka, India

Madhavaprasad CB
Professor and Head, Department of VPH&E, Veterinary College, Shivamogga, Karnataka, India

Prashanth S Bagalakote
Assistant Professor, Department of VPH&E, Veterinary College, Shivamogga, Karnataka, India

Arun SJ
Assistant Professor, Department of Veterinary Microbiology, Veterinary College, Gadag, Karnataka, India

Ganesh Udupa K
Professor and Head, Department of Veterinary Medicine, Veterinary College, Shivamogga, Karnataka, India

Ananthakrishna LR
Assistant Professor, Department of Veterinary Physiology and Biochemistry, Veterinary College, Shivamogga, Karnataka, India

Corresponding Author:
Shamanth Rakkith RN
M.V.Sc., Department of Veterinary Public Health and Epidemiology Veterinary College, Shivamogga, Karnataka, India

Quantification of biofilm forming ability of *Salmonella* species at different time interval

Shamanth Rakkith RN, Madhavaprasad CB, Prashanth S Bagalakote, Arun SJ, Ganesh Udupa K and Ananthakrishna LR

Abstract
The ability of 12 *Salmonella* isolates originating from animal, birds and environmental sources to form biofilms was investigated using 96-well flat microtitre plate assay. At 24-hours, none of the isolates formed biofilm, while most isolates were weak biofilm producers at 48 and 72 hours. As the biofilms has the ability to resist disinfectants, antimicrobials and contribute to virulence, has gained importance in food industries.

Keywords: Biofilm, microtiter, *Salmonella*

1. Introduction
Salmonella is one of the most common and one most important zoonotic foodborne pathogen responsible for food borne illness globally (Gharieb *et al*., 2015) [6]. *Salmonella* organism possesses virulence factors which help them to adhere to surfaces such as plastic, glass, stainless steel or rubber surfaces and form biofilm (Joseph *et al*., 2001) [8]. A biofilm is a population of microbial cells growing on a surface and enclosed in an amorphous extracellular matrix (Donlan, 2002) [5]. These biofilms act as a source of microbial contamination that leads to food spoilage and exhibit resistance to cleaning and sanitation (Bower and Daeschel 1999) [3]. Biofilm promotes the survival of the bacteria under low temperatures, heat, acidic pH, low nutrient conditions and antimicrobials (Steenackers *et al.*, 2012) [10]. Temperature is one of the most important environmental factors and they are formed in the range of 20-37 °C and also depends on nutrient availability and contact surface. (Stepanovic *et al*., 2003) [11]. The ability of bacteria to form biofilms has been estimated using various phenotypic methods like test tube method, microtitre plate test, congo red agar test (CRA) and colony count enumeration method. Among these, microtititre plate method was the simplest quantitative method to study the biofilms which allow for a numerical evaluation of the ability of bacteria to form biofilms. Considering these facts, the present study was aimed to detect biofilm-forming ability of *Salmonella enterica* and non-*enterica* subspecies in different time intervals.

2. Materials and Methods
A total of 12 isolates comprising of 6 *Salmonella* Typhimurium, 3 *Salmonela enterica* spp., 2 *Salmonela enterica* subspp *salamae* and 1 *Salmonela enterica* sub spp *diarizonae* were isolated from different sources *viz*. feces of animal and bird origin, cloacal swabs, soil, and floor swabs and were confirmed using PCR and were maintained in glycerol stock at Department of Veterinary Public Health and Epidemiology, Veterinary College – Shivamogga, Karnataka. These confirmed isolates were revived and selected for the present study.

2.1 Quantification of biofilms
The quantification of biofilms was performed in 96 well microtitre plates in brain heart infusion (BHI) broth as per Stepanovic *et al*., (2004) [12]. Each well of the microtiter plates were filled with 230 µl BHI broth. Overnight *Salmonella* culture of 20 µl was added into each well and each test isolates were made in triplicates. The wells containing BHI broth only was considered as negative control. The microtitre plate was kept for incubation aerobically at 37ºC for three different incubation periods (24 hrs, 48 hrs and 72 hrs). After incubation, the contents were discarded and the wells were washed with 300 µl sterile distilled water and fixed with 250 µl of methanol for 15 minutes and the contents were emptied and air-dried. Each well was stained with 250 µl of 1% crystal violet for 5 minutes and the excess stain was removed

by washing with running tap water and air dried. The attached cells were resolubilized with 250 µl of 33 % glacial acetic acid. The optical density (O.D) of each microtitre plate was measured using an ELISA reader (Biorad®) at 595 nm. Cut-off O.D. (O.D.c) was defined as three standard deviations above the mean O.D. of the negative control. Strains were classified as no biofilm producer [O.D. test ≤ O.D. control], weak biofilm producer [O.D. control < O.D. test ≤ (2 X O.D. control)], moderate biofilm producer [(2 X O.D. control) < O.D. ≤ (4 X O.D. control)] and strong biofilm producer [(4 X O.D. control) < O.D test].

3. Results

Biofilm formation of *Salmonella* species and subspecies was determined for different incubation time such as 24 hours, 48 hours and 72 hours. At 24 hrs, average O.Dc was 0.155 and *Salmonella* isolates CKF28, CCS5, SF47, PS8, CF59, SF81, CKF96, CKFS13, CF112, ES12, SFS17 and CKF143 obtained average O.D. value of 0.124, 0.161, 0.103, 0.110, 0.108, 0.35, 0.150, 0.093, 0.111, 0.102, 0.165 and 0.113, respectively. Since the O.D. value of all isolates was less than O.Dc, they were considered as non-biofilm formers.

At 48 hrs, average O.Dc was 0.175 and *Salmonella* isolates CKF28, CCS5, SF47, PS8, CF59, SF81, CKF96, CKFS13, CF112, ES12, SFS17 and CKF143 obtained average O.D. value of 0.201, 0.213, 0.209, 0.202, 0.214, 0.209, 0.204, 0.184, 0.180, 0.223, 0.211 and 0.196, respectively. Since the O.D. value of all isolates was more than the O.Dc but less than two times O.Dc, they were considered as weak biofilm formers. At 72 hours, average O.Dc was 0.197 and *Salmonella* isolates CKF28, CCS5, SF47, PS8, CF59, SF81, CKF96, CKFS13, CF112, ES12, SFS17 and CKF143 obtained average O.D. value of 0.245, 0.255, 0.261, 0.244, 0.247, 0.275, 0.234, 0.277, 0.286, 0.370, 0.266 and 0.293, respectively. Since the O.D. value of all isolates was more than the O.Dc but less than two times O.D. control, they were considered as weak biofilm formers, except for one *Salmonella* Typhimurium isolate ES12 (S10) that amplified ESBL gene (bla_{TEM}) was moderate biofilm producer, since the O.D value is more than two times O.Dc but less than four times O.Dc (Table 1 and Fig 1).

Table 1: Biofilm formation of *Salmonella* species / subspecies at different time interval

Sl. No.	Isolate No. and *Salmonella* species / subspecies / serotypes	Biofilm formation at different time interval											
		24 hours				48 hours				72 hours			
		No biofilm	Weak	Moderate	Strong	No biofilm	Weak	Moderate	Strong	No biofilm	Weak	Moderate	Strong
***Salmonella enterica* species**													
1.	*S.*Typhimurium (CKF28)	+	-	-	-	-	+	-	-	-	+	-	-
2.	*S.* Typhimurium (CCS5)	+	-	-	-	-	+	-	-	-	+	-	-
3.	*S.* Typhimurium (PS8)	+	-	-	-	-	+	-	-	-	+	-	-
4.	*S.* Typhimurium (CF59)	+	-	-	-	-	+	-	-	-	+	-	-
5.	*S. enterica* (SF81)	+	-	-	-	-	+	-	-	-	+	-	-
6.	*S.*Typhimurium T (CKF96)	+	-	-	-	-	+	-	-	-	+	-	-
7.	*S. enterica* (ES12)	+	-	-	-	-	+	-	-	-	-	+	-
8.	*S.*Typhimurium T (SFS17)	+	-	-	-	-	+	-	-	-	+	-	-
9.	*S. enterica* (CKF143)	+	-	-	-	-	+	-	-	-	+	-	-
***Salmonella* subspecies non-*enterica* group**													
1.	*salamae* (CKFS13)	+	-	-	-	-	+	-	-	-	+	-	-
2.	*diarizonae* (SF47)	+	-	-	-	-	+	-	-	-	+	-	-
3.	*salamae* (CF112)	+	-	-	-	-	+	-	-	-	+	-	-

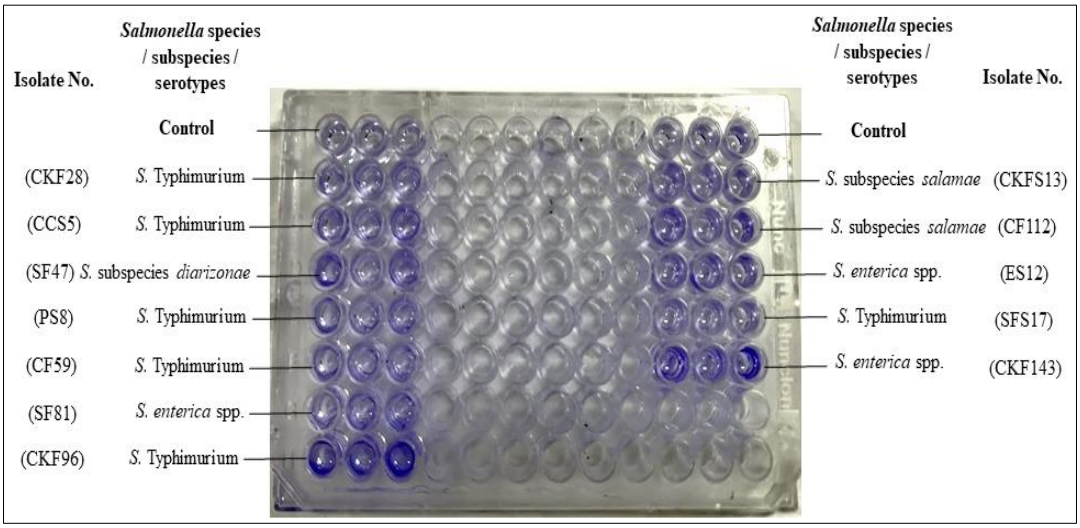

Fig 1: Biofilm formation of *Salmonella* species / subspecies

4. Discussion

There are many factors which affect the biofilm formation like nutrients level, pH, temperature, incubation period, ionic strength, culture concentration, *etc.*, The variation in the growth of biofilm can be due to difference in the composition of different media (Stepanovic *et al.*, 2004) [12] and 48-hours incubation time was suggested for biofilm formation of *Salmonella* species (Brendan and Ethan, 2005) [2].

Reza *et al.* (2018) [9] detected biofilm formation in 282 *Salmonella* isolates in nutrient broth out of which 98 (34.5%) isolates were strong biofilm producers, 168 (59.6%) were moderate and 16 (5.7%) isolates were weak biofilm producers, which is in contrast to the present study. Similarity to the present study, Agarwal *et al.* (2011) [1] showed that none of the *Salmonella* isolates formed biofilms at 24-hour incubation, while after 48 hours all isolates were found to produce biofilms in Luria-Bertani (LB) broth.

Another study by Jayaweera *et al.* (2021) [7] detected biofilm formation and found that 7 strong biofilm formers, 11 moderate biofilm formers, and 05 weak biofilm formers in diluted medium (TSB diluted 1:100) and there were only 04 moderate biofilm formers and 19 weak biofilm formers found in the TSB medium. Biofilms also acts as a source of contamination in food industries and also develop resistance against sanitizers that leads to substantial economic problems in food industries (Bridiera *et al.*, 2011) [4].

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