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QUANTITATIVE ANALYSIS OF BIOFILM FORMED BY *SALMONELLA* ENTERITIDIS BY USING DIFFERENT MEDIA AND SUBSTRATES

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Abstract- *Salmonella* Enteritidis biofilm was formed successfully *in vitro* by using five different media and substrate combination. The total biomass of the biofilm formed using different media and substrate varied considerably. The best biofilm was observed when the bacterium was grown in TSB in combination with 1% Chitin (4.749±0.1), while the least biofilm was formed in LB in combination with 1% Glass wool (1.539±0.02). The average of all the media irrespective of substrates revealed that in RPV maximum biofilm (4.068±0.144) was formed whereas, the least was formed in LB.

Keywords- Salmonella, Biofilm, LB, TSB, Chitin, Plastic, Glass wool, Medium

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Introduction

Salmonella is a major cause of enteric disease with manifestations ranging from gastroenteritis to septicemia and typhoid fever. It is an important member of the family *Enterobacteriaceae*. The genus *Salmonella* has two species *enterica* and *bongori*. *S. enterica* has been further sub divided into six subspecies viz., *enterica*, *salamae*, *arizonae*, *diarizonae*, *houtenae* and *indica* and there are > 2500 serovars of *Salmonella enterica* subspp *enteria* which have been recognized till now. In poultry, salmonellosis causes huge mortality in young birds and economic losses through reduced production in the adults [1].

A biofilm is a well-organized community of bacteria in which a bacterium attaches to any suitable surface and become "sessile". This adhesion to surface provides considerable help to the bacteria, such as protection from antimicrobial agents, exchange of nutrients, metabolites or genetic material from close proximity to other microorganisms [2-4]. It has been observed that both pathogenic and non pathogenic bacteria get incorporated into a biofilm over a period of time leading to formation of a very strong film. The biofilms formed on contact surfaces are thus a continuous source of contamination to whosoever coming in contact with them. Many bacteria which are of concern to veterinary professionals such as *Listeria*, *Pseudomonas*, *Campylobacter*, *E.coli, Klebsiella* and *Salmonella* are commonly associated with biofilm formation [5] and the biofilm forming capacity appears to be widespread among natural isolates of *S.* Enteritidis and *S.* Typhimurium, which aids in its establishment on environmental surface [6-7]. Thus, in the present study biofilm was formed using *S.* Enteritidis under in vitro conditions and the influence of various growth media and different substrates along with their combination helping in the formation of *S.* Enteritidis biofilm was evaluated.

Material and Methods

Procurement of Culture

Standard *S.* Enteritidis culture was procured from the Division of Bacteriology & Mycology, Indian Veterinary Research Institute, Bareilly, India and was maintained on Nutrient agar slant and was preserved at -20ºC on Nutrient agar slants containing 5% glycerol.

Formation of Biofilm

The formation of biofilm was done using five media viz., Luria Bertani broth (LB) (Hi Media, Mumbai), Nutrient broth (NB) (Hi Media, Mumbai), Tryptone soya broth (TSB) (Hi Media, Mumbai), Brain heart infusion broth (BHI) (Hi Media, Mumbai) and Rappaport vassiliadis medium (RPV) (Hi Media, Mumbai). The biofilm was formed on a sterile 12 well culture plate (Tarson, Kolkatta) by adding 3.68 ml of TSB, BHI, LB, NB and RPV individually in each well of the plate [Fig-1]. Later, 1% Chitin, 2% (Sigma, USA), Glass wool (SD Fine Chemicals, Mumbai), 5-6 chips Plastic chips (Polystyrene), 0.1% chitin were added column wise [Fig-1]. These plates are covered with lid and placed in an incubator for 12 h at 37ºC for sterility check. Later, 0.32 ml of 6 h grown *S.* Enteritidis culture from TSB was added into each well of above 5 formulated plates containing different media. All the plates were sealed and were placed in an incubator at 37ºC for 5 days undisturbed for the development of biofilm.

Collection of Biofilm

After 5 days of incubation the contents from the individual wells was aspirated and washed thrice with phosphate buffered saline (PBS)

gently so as to remove unattached bacterial cells. The biofilm phenotypes were collected with the help of a sterile swab by scrapping from any one wells each from each of five plates containing different medium and inoculated onto Hektoen Enteric Agar (HEA) (Hi Media, Mumbai) and the plates were allowed to get dried.

Crystal Violet Assay for Quantitation of Biofilm

The dried wells were initially fixed by adding methanol up to the brim of each well and incubated at room temperature. After incubating for 15 minutes methanol was discarded and the plates were dried. Crystal violet (1%) was added in each well and after five minutes the plates were washed with distilled water and allowed to get dry. Finally 33% glacial acetic acid in each well was added to measure the Optical Desnsity (OD) was measured at 570 nm (Tecan, The Netherlands) [8].

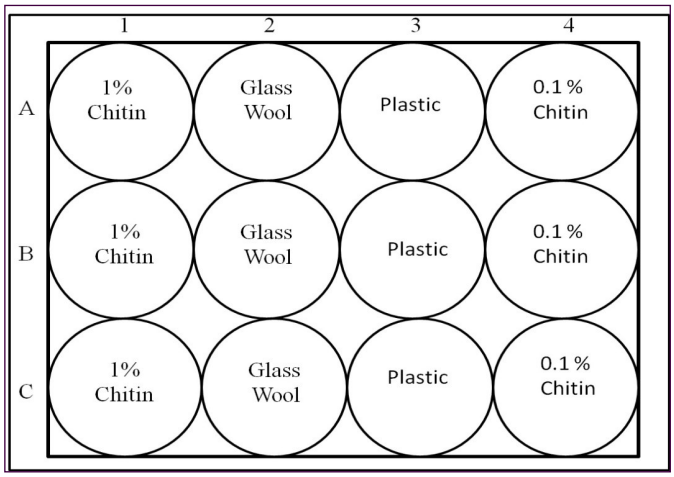

Fig. 1- Schematic representation of tissue culture plate used for biofilm formation

Statistical Analysis

The data was subjected to one way analysis of variance [9] performed using SPSS 9 [10].

Results and Discussion

Biofilm is a community of bacteria living under an organized system [11]. The objective of this study was to understand the influence of growth media along with different biofilm enhancing substances in the formation of biofilm by *Salmonella* Enteritidis and it was found that biofilm was formed successfully by using five different media and substrate combinations after five days of incubation.

It was observed that quantitatively maximum biofilm was formed when the bacterium was grown in TSB in combination with 1% chitin (4.749±0.1), while the least biofilm was formed when grown in LB with 1% glass wool (1.539±0.02) [Table-1]. The capability to successfully form biofilm is widespread among the common isolates of *Salmonella* Enteritidis and *Salmonella* Typhimurium [6, 7, 12-13] which was in agreement with our results. The above observations were similar with that of Stepanovic [14] where TSB was found most effective in promoting biofilm.

In another study Djordjevic [15] investigated the biofilm forming ability of 31 *Listeria monocytogenes* on food-processing surfaces and standardized a polyvinyl chloride (PVC) microtiter plate assay to form biofilm which was used successfully in our study in the formation of *Salmonella* biofilm. In this study, we found that there was inherent ability of the bacteria to undergo transformation into biofilm phenotype when exposed to appropriate environmental conditions which is similar to the study by Costerton [16] and Parsek and Fuqua [17] who independently evaluated formation of biofilm on a wide variety of surfaces including living tissues, medical devices and in the pipes which supply water and reported development of biofilm by the bacteria spontaneously.

When the overall biofilm formed in all the individual media irrespective of the substrate was evaluated, it was observed that biofilm was best formed in RPV (4.068±0.144) whereas was least formed in LB (2.95±0.233) [Table-2]. The above findings were in concurrence with [18] in which they found that when biofilm producing *S.* Enteritidis was pre incubated in media containing increasing levels of glucose concentration, the levels of both cytoplasmic glycogen and biofilm rose correlatively.

When the overall biofilm formed in all the substrates irrespective of the media was evaluated it was observed that 1% chitin had the best biofilm enhancing ability (4.749±0.1) while the plastic chips had the minimum biofilm enhancing ability (2.831±0.11) (Table 2). The results were in concurrence with the studies of Divya and Selvam [19], where they successfully produced *Escherichia coli* O157:H7 employing 0.08 per cent Tryptic soya broth (TSB) with 0.3 per cent chitin. In an earlier study [20] it was reported that surfaces seem to play a major role in the survival of the microbial cells and adhesion of bacterium to surfaces in biofilm similar to what we observed in our study i.e. incubation of bacteria with varied substrate yielded different quantity of biofilm. The exact reason for the enhanced formation of biofilm using chitin is not known and needs to be studied further.

Table 1- Combined Mean (M) ± Standard Error (SE) of the OD of the biofilm formed in various media and substrates

SNo.	Media	Substrates			
		1% chitin	0.1% chitin	Glass wool	Plastic chips
1	RPV	4.769±0.255^{a}	3.62±0.053^{b}	4.639±0.271^{a}	3.244±0.094^{bceg}
2	TSB	4.824±0.288^{a}	3.595±0.023^{b}	2.874±0.102^{gih}	3.08±0.045^{dik}
3	BHI	4.8±0.186^{a}	3.504±0.25^{bk}	2.855±0.085^{dgh}	3.541±0.17^{bce}
4	NB	4.557±0.269^{a}	3.604±0.113^{b}	4.432±0.064^{a}	2.522±0.217^{h}
5	LB	4.793±0.116^{a}	3.7±0.128^{b}	1.539±0.02^{ej}	1.768±0.044^{fj}

No common superscript indicates significant difference (P<0.05)

RPV: Rappaport Vassiliadis Medium; TSB: Trypticase Soy Broth; BHI: Brain Heart Infusion; NB: Nutrient Broth; LB: LuriaBertani

Table 2- Mean (M) ± Standard Error (SE) of the OD of the biofilm formed using different media and substrates

SNo.	Medium	M ± SE	Substrate	Mean ± Standard Error
1	RPV	4.068±0.144^{a}	1% chitin	4.749±0.1^{a}
2	TSB	3.593±0.148^{b}	0.1% chitin	3.604±0.059^{b}
3	BHI	3.675±0.148^{b}	Glass wool	3.268±0.182^{c}
4	NB	3.779±0.163^{b}	Plastic chips	2.831±0.11^{d}
5	LB	2.95±0.233^{c}		

No common superscript indicates significant difference (P<0.05)

RPV: Rappaport Vassiliadis Medium; TSB: Trypticase Soy Broth; BHI: Brain Heart Infusion; NB: Nutrient Broth; LB: LuriaBertan

Conclusions

Thus, from the above study it could be concluded that biofilm was successfully formed by using five different media in combination with various substrates. It could be further concluded 1% chitin had the best biofilm enhancing qualities (4.749±0.1) while the plastic chips showed the minimum (2.831±0.11) biofilm enhancing.

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Conflict of Interest: Author declares that, there is no conflict of interest.

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