

Original Research Article

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Quantification of Biofilm Formation in Indian Isolates of *Salmonella enterica*

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ABSTRACT

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A total of 60 *Salmonella* isolates (43 poultry origin, 17 farm animal origin) received from various parts of the country were used in the current study. The objective is to study the biofilm forming potential of the isolates using microtitre plate assay. Study revealed that 38% of the isolates were moderate biofilm producers and 37% were strong biofilm formers. As biofilm contribute to virulence and antimicrobial resistance of the organism, the study remains significant in food safety aspect.

Introduction

Non typhoidal *Salmonella* spp., is a gram negative bacterial pathogen associated with gastrointestinal tract of farm animals like cattle, pigs and poultry (WHO, 2013). The organism transmits to human via faeco-oral route and causes food borne illness. Non typhoidal salmonellosis outbreaks occur worldwide comprising about 93 million cases of gastroenteritis and 1,55, 000 deaths annually (Majowicz *et al.*, 2010). Various virulence factors of *Salmonella* spp., which helps in adhesion and invasion are responsible

for the pathogenicity. In addition, *Salmonella* spp., also possess the ability to form biofilms. Biofilms are the aggregates of bacterial species inside an extracellular polysaccharide matrix. Inside biofilms, bacteria will be in natural stationary phase (Lopez *et al.*, 2010) and their physiology will be different from that of their planktonic counterparts. This helps the bacteria to escape from host immune response. It has been studied that *Salmonella* spp., could able to form biofilms in various surfaces such as plastic, stainless steel, glass, rubber, gall stones, animal cells and plants (Steenackers *et al.*, 2012). The bacteria

residing inside biofilms thus protect themselves from UV light, disinfectants, antimicrobial agents etc (Lianou and Koutsoumanis, 2012). Therefore biofilm indirectly contributes to the organisms' virulence and antimicrobial resistance. Various phenotypic methods like test tube method, microtitre plate test, congo red agar test (CRA) and colony count enumeration method have been widely used to study the biofilms. Apart from this, microscopic techniques like Confocal laser microscopy, Scanning Electron Microscopy, Optical coherence tomography and genotypic methods involving relative expression of biofilm associated genes in quantitative PCR have also been used to study biofilms (Merino *et al.*, 2017). Among these, microtitre plate method was the simplest quantitative method to study the biofilms. In the current study, biofilm quantification using microtitre plate method has been done to 60 *Salmonella enterica* isolates.

Materials and Methods

Bacteria

60 *Salmonella enterica* isolates stored in buffered nutrient slant at National Salmonella Centre, IVRI, Bareilly were used in the current study. The cultures were revived in BHI broth and streaked on Hektoen Enteric plates. Among the 60 bacterial isolates, 43 were from poultry origin and 17 were from farm animals like sheep, goat, pig and cattle. The serovars used for the study includes Typhimurium, Kentucky, Virchow, Enteritidis, Welteverden, Heidelberg, Bovismorbificans, Rough Salmonella, Eastbourne, Dublin, Gallinarum, Haifa, Sandiego, Paratyphi B, Berto and Indiana.

Biochemical test

The isolates were confirmed biochemically by streaking them in Triple Sugar Iron agar

(Himedia, India). The TSI agar contains sugars such as 0.1% glucose, 1% sucrose and 1% lactose, ferric ammonium salts as hydrogen sulphide indicator and phenol red as pH indicator.

Polymerase chain reaction for *invA* gene

The PCR for *invA* gene was performed as per Galan *et al.*, (1992) with certain modifications. The reaction mixture was optimized to contain 12.5 µL of 2X PCR buffer, 10 pmol of each forward and reverse primers (Table 1) and 5 µl of bacterial lysate prepared by boiling and snap chilling method and nuclease free water was added to make up the volume to 25 µl. The PCR cycling condition comprised of an initial denaturation at 94°C for 1 min, primer annealing at 50°C for 1 min, elongation at 72°C for 1 min and finally a single step extension at 72°C for 7 min. The PCR products were analysed by running in 1.5 % agarose gel electrophoresis gel.

Biofilm assay

The quantification of biofilms was done in 96 well microtitre plates as per Stepanovic *et al.*, (2004) with little modifications. Various serovars of *Salmonella enterica* were used in the current study. Briefly, 80 µL of the overnight grown culture was mixed with 920 µl Luria Bertani (LB) broth and 250 µl of the later in triplicate was pipetted in each well of the plate.

The plates were covered with aluminium foil and incubated at 37° C for 48 hrs. After 48 hrs, the contents were poured off and washed with 250 µl of sterile distilled water. The biofilms are fixed with methanol @ 250 µL /well for 15 min. The contents were poured off and air dried. Staining is done with crystal violet @ 250 µL/well for 5 min followed by washing with sterile distilled water. Resolubilization of the dye was done in 33 %

glacial acetic acid @ 250 µl/ well. The absorbance was measured at optical density of 570 nm. Three wells containing LB broth without any culture is considered as control.

Classification of test isolates based on biofilm intensity

The isolates are classified into various categories based on the optical density (OD) of the control as per Stepanovic *et al.*, (2004) as follows:

OD_{control} = 3 (Standard deviation) above the mean OD of negative control

- 1) No biofilm producer = OD_{test} ≤ OD_{control}
- 2) Weak biofilm producer = OD_{control} < OD_{test} ≤ (2 × OD_{control})
- 3) Moderate biofilm producer = (2 × OD_{control}) < OD_{test} ≤ (4 × OD_{control})
- Strong biofilm producer = (4 × OD_{control}) < OD_{test}

Results and Discussion

Bacterial growth

All the *Salmonella enterica* isolates produced transparent green or bluish green colonies with black centres.

Biochemical test

After 18-24 h of inoculation, the organisms produced alkaline slant and acid butt with black precipitate and gas production. The organism could able to ferment only glucose to CO₂, with H₂S production.

Molecular characterization

PCR amplification of the isolates targeting *invA* gene revealed specific amplification at 284 bp on agarose gel electrophoresis.

Biofilm assay

Among the 60 isolates, 9 isolates were observed as non biofilm producers, 6 isolates as weak biofilm producers, 23 as moderate biofilm producers and 22 as strong biofilm producers.

Among the 43 poultry isolates, majority are moderate biofilm producers (17) followed by strong biofilm producers (15). Among the 17 farm animal isolates, majority were strong biofilm producers (7) followed by moderate producers (6). The tabular form of the biofilm assay results were presented in Table 2.

Table.1 Primer sequence

S.No	Gene	Primer sequence	Product size
1	invA F invA R	GTGAAATTATCGCCACGTTCTGGGCAA TCATCGCACCGTCAAAGGAACC	284bp

Table.2 Biofilm assay

Animal	Non biofilm	Weak	Moderate	Strong	Total
Poultry	6	5	17	15	43
Farm animals	3	1	6	7	17
Total	9 (15%)	6 (10%)	23 (38.33%)	22 (36.67%)	60

Fig.1 Bacterial culture in Hektoen enteric plate

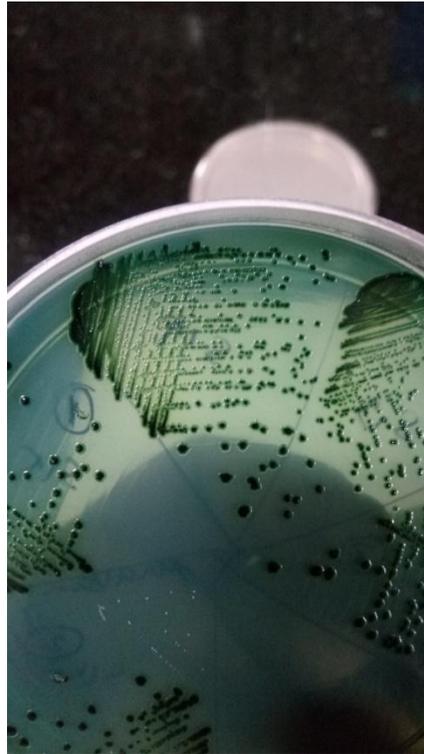
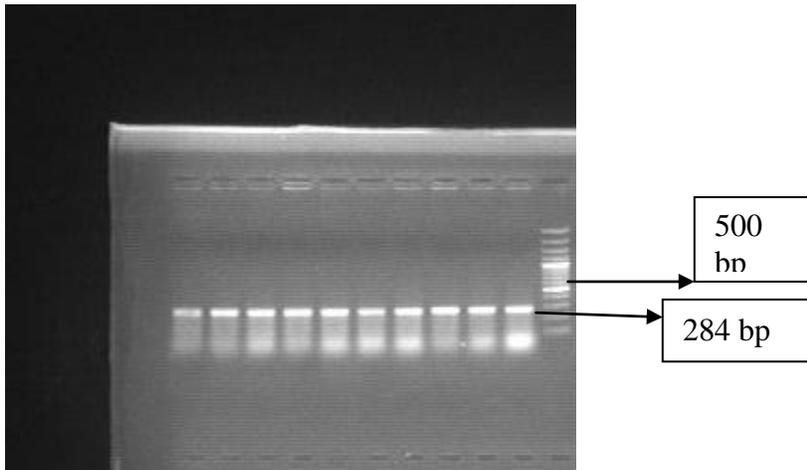


Fig.2 Biochemical test in TSI agar

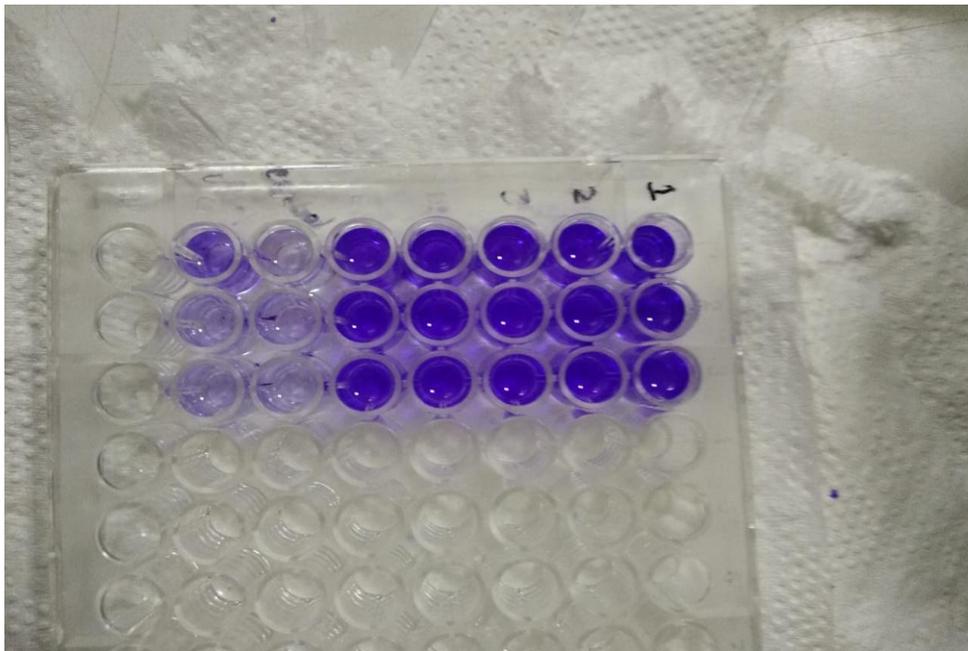


Fig.3 *invA* gene PCR



Lanes 1-10: *Salmonella* isolates
Lane 11: 100 bp ladder

Fig.4 Biofilm assay in 96 wellled microtitre plate



Under in vitro conditions, environmental conditions such as temperature, pH, osmolarity, media composition etc affect biofilm production. It has been proven nutrient less medium favours high quantity of biofilm formation in *Salmonella* sps., Moreover the serovars have no significant

influence on biofilm production (Stepanovic *et al.*, 2004). Therefore, in the current study serovars were not taken into consideration while analyzing the results. It is of more obvious from the current study that around 60% of the isolates were moderate to strong biofilm producers. This is of high significance

as biofilms contribute to both virulence and antibiotic resistance of the bacteria. Moreover, biofilms contribute to the survival of bacteria in meat and other food products. Around 40% of the poultry isolates and 35% farm animal isolates were moderate biofilm producers followed by 37.5 % and 40% strong biofilm producers in poultry and farm animal isolates, respectively. Previous reports revealed that around 50% *Salmonella* isolates from poultry origin were biofilm producers (Marin *et al.*, 2009). The biofilm formation provides an added advantage of persistence of bacterial species in spite of regular cleaning and disinfection. This possesses risk to food safety and in turn human health. Therefore, the current study is of importance in the public health context.

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