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Biofilm Formation and Associated Genes in *Listeria Monocytogenes*

S. R. Warke, V. C. Ingle, N. V. Kurkure, P. A. Tembhurne, Minakshi Prasad, S.P. Chaudhari and S. B. Barbuddhe

Department of Veterinary Microbiology and Animal Biotechnology

Nagpur Veterinary College, MAFSU, Nagpur (Maharashtra)

Corresponding Author: : shubhangi_warke@rediffmail.com

Abstract

Listeria monocytogenes, an opportunistic food borne pathogen can cause serious infections in immunocompromised individuals. *L. monocytogenes* is capable of producing biofilm on the surface of food processing lines and instruments.The biofilm transfers contamination to food products and impose risk to public health. In the present study biofilm producing ability of *L. monocytogenes* isolates were investigated phenotypically and genotypically by microtiter assay and multiplex PCR, respectively. Out of 38 *L. monocytogenes* isolates 14 were recovered from animal clinical cases, 12 bovine environment and 12 from milk samples. A total of 3 (21.42%) clinical, 2 (16.66%) environment and 3 (25%) milk samples respectively, revealed biofilm production in microtiter assay. Cumulative results showed that 23 (60.52%) out of 38 strains of *L. monocytogenes* were positive for *luxS* and *fla*A gene and 1 (2.63%) was positive only for the *flaA* gene.

Key Words: Biofilm,*Listeria monocytogenes*, Microtiter plate assay, PCR.

Introduction

Listeriosis is one of the important bacterial zoonotic infections caused by *L. monocytogenes* (Baylegen *et al*., 2004). *L. monocytogenes* may form biofilm which is characterised as community of bacteria living in organized structures at a liquid interface (Davies, 2003). *Biofilms allow bacteria to better resist environmental stresses, such as dehydration, treatment with antimicrobial and sanitizing agents (*Lewis, 2001, Folsom and Frank, 2006*).* Biofilms could serve as a source of product contamination and as reservoir for pathogenic or spoilage bacteria (Sommer *et al*., 1999). Ability of *L. monocytogenes* to form biofilm is considered as an important virulence determinant influencing its pathogenicity. The ability of biofilm formation of *L. monocytogenes* on surfaces within the food processing environment may be one of the important factor in their survival (Holah *et al.,* 2004; Chae *et al.,* 2006). Most of the time, in the food processing environment, *L. monocytogenes forms biofilms on abiotic surfaces in association with other bacteria, such as Pseudomonas spp (*Fatemi and Frank, 1999*).* The studies have described the role of *fla*A and *lux*S systems of *L. monocytogenes* in surface attachment and biofilm formation (Sela *et al*., 2006). The flagella produced by flagellar gene *fla*A are implicated as surface adhesions in *L. monocytogenes* (Vatanyoopaisarn *et al*., 2000). It also facilitates attachment of bacteria to both biotic (Piette and Idziak, 1991) and abiotic (O'Toole

and Kolter, 1998) surfaces. The *lux*S gene encodes *S*-ribosylhomocysteine, which serves as a precursor of autoinducer 2 (AI-2) and play a role in biofilm formation (Vendiville *et al*., 2005).

In India, a little work has been done on genotypic characterization of *L. monocytogenes* and its biofilm forming genes *lux*S and *fla*A. Hence the present study was planned to *standardize a Microtiter plate assay and a PCR protocol for assessing in-vitro biofilm formation ability and detection of biofilm associated genes (flaA and luxS) in L. monocytogenes*.

Materials and Methods

Out of 625 different samples a total 38 isolates of *Listeria monocytogenes* were identified. Out of these 38 samples, 14 from animal clinical cases (vaginal swabs), 12 from milk samples and 12 from environment of bovine farm i.e. feed, fodder, water, manure/fertilizers, silage, soil and drainage were used for the present study. These isolates were maintained on Brain Heart Infusion (BHI) agar at 4°C for biofilm formation assay. Isolates were transferred from BHI media to 5 ml of Tryptone Soy Broth (TSB) and incubated for 18 h at 30°C. The microtitre plate biofilm screening assay was done as described by Harvey *et al* (2007). To test significant differences between the isolates of different origin used and within replicates, the ANOVA test was applied.

Genotypic identification of *luxS* and *fla*A genes in *Listeria monocytogenes* by PCR.

The primers for detection of *lux*S and *fla*A genes were designed and synthesized by Sigma Aldrich, Bangalore (India). For *lux*S (lmo 1288) and *fla*A (lmo 0689) two sets of primers with forward primer 5'GGA AAT GCC AGC GCT ACA CTC TTT 3', reverse primer 5'ATT GCA TGC AGG AACTTC TGT CGC 3' and forward 5'GCG CAA GAA CGT TTA GCA TCT GGT 3' and reverse 5' TTG AGT AGC AGC ACC TGT AGC AGT 3' with the amplicons size of 208 and 363 bp respectively were designed. The genomic DNA was isolated from all the isolates by standard phenol chloroform method. Multiplex PCR targeting *lux*S and *fla*A genes of *Listeria monocytogenes* was standardized.

Reaction mixture was prepared as 50 µl reaction volume with the composition of 5.0 µL of 10X PCR buffer, 2.0 µl of 25 mM $MgCl_2$, 2.0 µl of 10 mM dNTP mix, 1 µl (10 pmol) each of forward and reverse primer for each gene, 0.25 µl of 5 IU/ µl Taq DNA polymerase, 1.5 µl of template DNA and sterilized milli Q water to make up the final reaction volume 50 µl.The cycle conditions included an initial denaturation at 94°C for 2 min. followed by 35 cycles each of 30 second denaturation at 94°C, 30 sec annealing at 58°C and 1 min. extension at 72°C. It was followed by final extension of 7 min. at 72°C. After the reaction, PCR products were visualized in 1.0% Agarose gel electrophoresis.

Results and Discussion

Biofilm formation by pathogenic microorganisms is of immense significance to food processing industries because *L. monocytogenes* is one of the important food-borne pathogen capable of colonizing food processing environments (Kumar *et al*., 2009) In the present study microtiter plate assay showed only 3 (21.42%) isolates from animal clinical cases, 2 (16.66%) from bovine environment and 3 (25.0%) from milk samples as phenotypically biofilm producer. On the other hand PCR studies indicated that 7 (50.14%) isolates from animal clinical cases, 8 (66.66%) from bovine environment and 8 (66.66%) from milk samples showed presence of biofilm associated *lux*S and *fla*A genes. However, one isolate (7.14%) recovered from animal clinical cases showed the presence of *fla*A gene alone.On comparison it was observed that out of 3 isolates recovered from animal clinical cases which were positive for biofilm formation by Microtiter plate assay, 2 were found to harbor *fla*A and *lux*S gene and one was found positive for only *fla*A gene. Similarly, 2 isolates obtained from bovine environment and 3 from milk were found to carry *fla*A and *lux*S gene together.

There was no significant difference between the amounts of biofilm formed by each strains ($P< 0.05$). All these strains showed a slight rise in the quantities of attached cells over 48 and 72 hours (Figure 1). No reduction in the amount of biofilm was observed during testing hours. The observed

OD $_{595}$ nm values ranged between 1.5 to 2.5 nm after 72 hours (Figure 1). These finding are in concurrence with the finding of previous study in that 10 *L. monocytogenes* isolates were able to form biofilm after 24 h at 20°C with variation in capability of biofilm formation. However, biofilm formation of all strains showed a slight rise in the quantities of attached cells over 48 and 72 hours (Fouladynezhad, *et al*., 2013). In another study Adetunji and Adegoke(2008) observed that 40 *L. monocytogenes* strains produced biofilms after 48 and 72 hours incubation at 37°C but no biofilm formation was observed at 24 hours. Similarly Harvey *et al.,* (2007) reported that out of 138 *L. monocytogenes* strains 127 (92.0%) were weak, 9 (6.5%) as moderate and only 2 (1.5%) were strong biofilm formers. The biofilm formation is also dependent on surface and it varies from surface to surface as on comparison of surfaces for 44 *L. monocytogenes* strains . Bonaventura *et al,*(2007) reported that biofilm formation was significantly higher on glass at 4,12 and 22 °C as compared to polystyrene and stainless steel surfaces. However, reverse trained was observed at 37°C.

In the present study there was no significant variation observed in biofilm forming ability in isolates of different origin. It is in contrast to the findings of Barbosa *et al* (2013) who reported significant difference (P< 0.01) between clinical and food isolates and at 37°C for 24 hours. Most food isolates of *L. monocytogenes* were classified as weak or moderate biofilm formers, whereas all the clinical isolates were strong biofilm producers (Barbosa *et al*., 2013). The use of culture media also affects the biofilm forming ability of *L. monocytogenes* (Dubravka *et al*, 2007). On comparison of three different culture media i.e., tryptone-soy broth with yeast extract (TSB-YE), brain heart infusion (BHI) and 1/20 diluted tryptone-soy broth with yeast extract (1/20 TSB-YE), the highest OD values were observed in TSB-YE media at 37°C.Thus lower percentage of biofilm producing isolates in present study might be attributed to media used for the study.

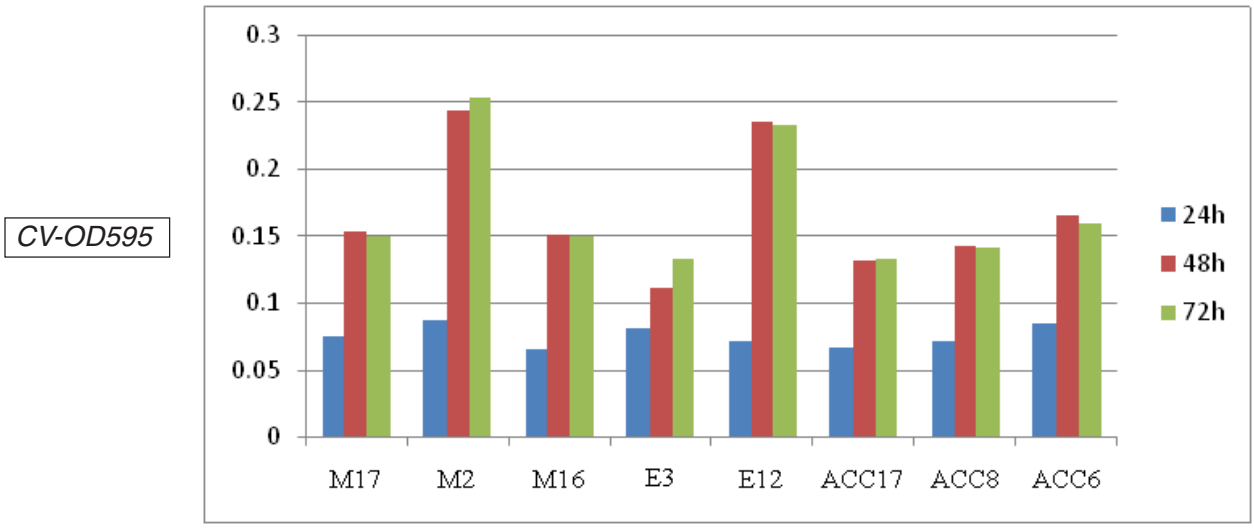

Figure1: Listeria monocytogenes showing biofilm formation at different intervals strains

Multiplex PCR was standardized for the detection of *flaA* and *luxS* genes in *L. monocytogenes*. The primer set for *flaA* and *luxS* allowed the positive amplification of 363 bp and 208 bp products respectively in test strains of *L. monocytogenes* (Figure 2). Cumulative results showed that 23 (60.52%) out of 38 strains of *L. monocytogenes* were positive for *luxS* and *fla*A gene and 1 (2.63%) was positive only for the *flaA* gene. None of the strain was positive for *luxS* gene alone (Table 1). Sela *et al.* (2006) observed that an intact *lux*S gene is associated with repression of components required for attachment and biofilm formation. Similarly, Katherine *et al*. (2010) reported the first evidence of positive role of *prf*A gene on extracellular biofilm formation.

The present study also indicate that all the *luxS* and *flaA* positive strains could not exhibit biofilm

Table 1:- Frequency of virulence-associated genes in *Listeria monocytogenes* isolates recovered from different sources by multiplex PCR

Source	No. of isolates	Amplified PCR products of virulence associated genes detected in *Listeria monocytogenes* isolates		Microtiter Plate assay
		***flaA* gene**	***flaA and luxS* gene**	
Animal clinical cases	14	1	07	3
Bovine environment	12	-	08	2
Milk	12	-	08	3
Total	**38**	1 (2.63%)	23(60.52%)	8(21.05%)

formation on microtiter plate. This may possibly due to that some isolates are genotypically positive for *fla*A and *lux*S gene, but phenotypic expression of genes could not occur in microtiter plate assay. This suggests that though the bacterium had the biofilm associated genes *flaA* and *luxS*, the genetic ability of *L. monocytogenes* for biofilm production might have been altered by environmental factors (Bonaventura *et al.,* 2007). However, such comparative studies on biofilm production and association of virulence genes have not been carried out.

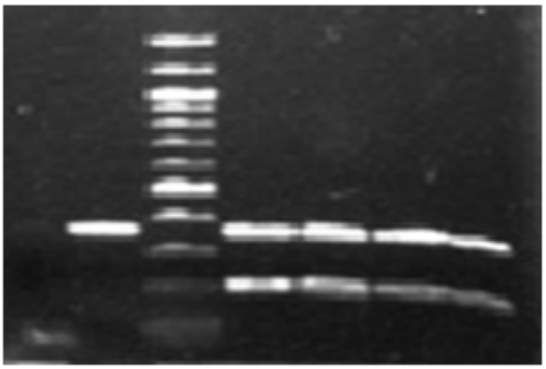

Figure 2: Amplification of *luxS* and *flaA* gene of *L. monocytogenes* by multiplex PCR.

Lanes: L: DNA ladder (100bp); 1- Amplicon of *flaA* (363 bp) gene; 2-5: Amplicon of *luxS* (208 bp) and *flaA* (363bp) genes of *L. monocytogenes*.

Conclusion

The *L. monocytogenes* is a biofilm producer organism. Isolates of *L. monocytogenes* recovered from different sources showed positive correlation between biofilm formation by Microtiter plate assay and virulence marker genes (i.e. *fla*A and *lux*S) associated with biofilm formation. PCR has better sensitivity as compared to Microtiter Plate Assay for the detection of biofilm formation for *Listeria monocytogenes*. Further research is required to find an effective way to clean the surfaces and also effective sanitization methods to completely remove this bacterium.

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Conflict of Interest: All authors declare no conflict of interest.

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