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Biofilm Quantification in *Listeria monocytogenes* 4B Serotype isolated from Animals of Gujarat State, India

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ABSTRACT

The purpose of this study was to assess slime production and quantification of biofilm in a set of well-characterized *Listeria monocytogenes* strains isolated from healthy and clinically affected animal. In all 28 strains of *L. monocytogenes* belonging to serotype 4b having proved *in vitro* pathogenicity potential is included in the study. Slime production was determined by cultivation of the organisms on Congo red agar medium, while quantification of biofilm was performed with the help of microtitre plate assay. Out of 28 isolates, 22 (78.57 %) strains of *L. monocytogenes* produced slime. The rest of the 6 (21.43 %) isolates were negative for slime production. For biofilm production, out of the 28 strains, 5 (17.86 %), 18 (64.29 %), and 5 (17.86 %) were found moderate, weak and negative, respectively. Strains belonging to *Listeria monocytogenes* serotype 4b showed inconsistent results regarding biofilm production. Biofilm productivity exhibited profound intra-strain variations irrespective of source of isolation. As *L. monocytogenes* are biofilm producers, this increases the probability of occurrence of animal and human infection. Further, as *L. monocytogenes* produces biofilm, infections caused by this bacterium may be underestimated because diagnoses in the presence of biofilm are difficult.

HIGHLIGHTS

- *Listeria monocytogenes* serotype 4b produces slime that may increase the occurrence of animal and human infections.
- *Listeria monocytogenes* serotype 4b showed inconsistency in biofilm production.

Keywords: Animals, Listeria monocytogenes, biofilm

Listeriosis is one of the important bacterial diseases of animals and a zoonosis with a broad distribution; it has considerable economic and public health impact. *Listeria monocytogenes*, a facultative intracellular pathogen responsible for Listeriosis, has been isolated from healthy and diseased animals, birds and human beings. It is a well-known cause of abortion, encephalitis and septicemia both in animals and human beings. Since the bacterium is ubiquitous in nature, found in soil and vegetation, it is easily contracted and transmitted to animals and contaminate process of food production (Matle *et al*., 2020). In particular, the psychrotropic nature of organism allows replication in refrigerated condition, ready-to-eat food products that have been contaminated during processing and packaging, resulting in food-borne outbreaks that are characterized by widespread distribution and relatively high mortality rates.

When organized as biofilm, the self-produced extracellular polymeric matrix gives extra protection to bacteria from harsh environmental conditions such as desiccation, nutrient deprivation, or disinfectant treatment (Bridier *et al*., 2011; Esbelin *et al*., 2018). Within a biofilm, bacteria become attached to a surface where they exist in complex communities that are able to interact with each other

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through intracellular communication and thus rapidly adapt to changing environments. Biofilm formed in animal environments was of special significance as it has the potential to act as the chronic source of microorganism that may lead to transmission of diseases. Moreover, exposure to a stress factor can provide cross-adaptation to subsequent exposure to other stresses (Bergholz *et al*., 2012). Complex mechanisms regulate bacterial sessile growth and biofilm formation represented by adhesion, maturation and dispersal steps, each affected by intrinsic and extrinsic factors (Matle *et al*., 2020).

Perusal of literature suggest that there is scanty of research about role of bacterial biofilms in animals especially under Indian conditions, but they are believed to be involved in many diseases such as pneumonia, liver abscesses, enteritis, wound infections and mastitis infections (Melchior *et al*., 2006a,b). These infections can be caused by environmental organisms, such as *P. aeruginosa*, *Staphylococcus aureus* etc. that are commonly found in wound infections, as well as by species of bacteria that constitute part of the normal microflora of animals. Through a combination of endogenous and exogenous factors these generally harmless commensals may become pathogenic. And considering extent and rate of bacterial attachment, it has been seen that microorganisms get attached to more rapidly to hydrophobic and nonpolar surfaces as Teflon and other plastics rather than to glass and other materials having hydrophilic properties (Choudhary *et al*., 2020). These plastics are nowadays frequently used in dairy industry under Indian conditions. Biofilm formation in microtiter plates is certainly the most commonly used method to grow and study biofilm. This simple design is very popular due to its high-throughput screening capacities, low cost, and easy handling (Thibeaux *et al*., 2020). The microtitre plate has the advantage of analyzing adhesion of multiple bacterial strains or growth conditions within each experiment. So, present study was carried out in a set of well-characterized *L. monocytogenes* strains for assessing biofilm detection and quantitation with the help of microtitre plate assay. The strains included clinical and animal sources.

MATERIALS AND METHODS

Ethical approval

As per the Committee for the Purpose of Control and Supervision of Experiments on Animals guidelines, a study involving collection of clinical samples under field conditions does not require approval of Institute Animal Ethics Committee. Blood samples were collected by licensed veterinarians as per standard sample collection methods without any harm or stress to the animals.

Procurement of standard bacterial culture

The standard strain of *L. monocytogenes* 4b (MTCC 1143) used in the study was obtained from the Microbial Type Culture Collection and Gene Bank, Institute of Microbial Technology, Chandigarh, India. The well-characterized strains of *L. monocytogenes* isolated from different health status of animals and sources are shown in Table 1 and Table 2. All the 28 strains are maintained in Department of Veterinary Microbiology, College of Veterinary Science & Animal Husbandry, Anand (India).

Table 1: Health status of animals from which *L. monocytogenes* strains were isolated

Sl. No.	Source of Isolation		*L. monocytogenes*
1	Apparently healthy Sheep	Faecal Samples	7
2	Cattle and Buffalo with reproductive tract disorders	Deep vaginal Swabs	9
		Faecal Samples	2
		Milk Samples	4
3	Lactating Animals	Faecal Samples	1
		Feed Samples	1
4	Clinical Mastitis	Mastitic Milk	3
5	Apparently healthy Zoo Animals	Fecal Samples	1

Table 2: Isolation of *L. monocytogenes* from Animals' Sources

Isolate No.	Animal source of Isolation	Source of Sample	Isolate No.	Animal source of Isolation	Source of Sample
LM1	Sheep	Feces	LM15	Cattle	Vaginal Swab
LM2	Sheep	Feces	LM16	Cattle	Vaginal Swab
LM3	Sheep	Feces	LM17	Cattle	Vaginal Swab
LM4	Sheep	Feces	LM18	Cattle	Vaginal Swab
LM5	Sheep	Feces	LM19	Cattle	Feces
LM6	Sheep	Feces	LM20	Cattle	Milk
LM7	Sheep	Feces	LM21	Cattle	Milk
LM8	Buffalo	Feces	LM22	Bufffalo	Milk
LM9	Buffalo	Feces	LM23	Cattle	Milk
LM10	Buffalo	Vaginal Swab	LM24	Environment	Feed
LM11	Buffalo	Vaginal Swab	LM25	Cattle	Milk
LM12	Buffalo	Vaginal Swab	LM26	Cattle	Milk
LM13	Buffalo	Vaginal Swab	LM27	Cattle	Milk
LM14	Buffalo	Vaginal Swab	LM28	Zoo animal	Feces

Characterization of *L. monocytogenes*

All the 28 strains were characterized for haemolysis activity, Christie Atkins Munch Peterson (CAMP) test (Annonymous, 1994), Phosphatidylinositol-specific Phospholipase C (PI-PLC) assay (Leclercq, 2004), Phosphatidylcholine-Specific Phospholipase C (PC-PLC) assay (Coffey *et al.,* 1996). All the strains were found positive for aforementioned characters.

All the 28 strains were serotyped as *L. monocytogenes* 4b using multiplex PCR assay following the methodology as described by Doumith *et al*. (2004) with suitable modifications.

Slime production assay

Slime production of the isolates was determined by cultivation of the organisms on Congo red agar medium as per the method described by Freeman *et al*. (1989). Each strain was streaked on the Congo red agar medium and incubated aerobically at 37ºC for 24 h followed by further incubation at room temperature (25ºC) for 48 h. The production of rough black colonies by the strains indicated production of slime.

Quantification of biofilm

Quantification of biofilm production in plastic microtitreplate was based on the previously described method of Stepanovic *et al*. (2004) with slight modification as follows. The wells of a sterile 96-well flat-bottomed polystyrene microtitreplate (Laxbro Ltd. India) were used for the test. The test organisms were grown in congo-red broth at 37 ºC for 18 h incubation. The microtitreplate were filled with 230 µl of the congo-red broth. A quantity of 20 µl of overnight bacterial culture of the test isolates was added into each well. Each isolates was tested in triplicate. The negative control wells contained broth only. The plates were incubated aerobically for 24 h at 35ºC. The content of the plate was then poured off and the wells washed three times with 300 µl of sterile distilled water. The remaining attached bacteria were fixed with 250 µl of methanol per well, and after 15 min microtitre plates were emptied and air-dried. The microtitre plates were stained with 250 µl per well of Crystal violet used for Gram staining for 5 min. Excess stain was rinsed off by placing the microtitre plates under running tap water. After the microtitre plates were air dried, the dye bound to the adherent cells was resolubilized with 250 µl of 33% (v/v) glacial acetic acid per well. The optical density (O.D.) of each well was measured at 570 nm using an automated Multiscan EX reader (Thermo Electron Corporation Ltd, Navi Mumbai, India).

Mean O.D of the test isolates tested in triplicates was taken as final O.D of the test isolates. Based on the O.D.

produced by bacterial films, strains were classified into the following categories: no biofilm producers, weak, moderate or strong biofilm producers, as described by Stepanovic *et al*. (2004). Briefly, the 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 follows: O.D. < O.D.c = no biofilm producer, O.D.c < O.D. < (2 × O.D.c) = weak biofilm producer, (2 × O.D.c) < O.D. < (4 × O.D.c) = moderate biofilm producer and (4 × O.D.c) < O.D. = strong biofilm producer.

RESULTS AND DISCUSSION

The objective of this study was to detect slime and biofilm formation from strains that were characterized has having pathogenic potential by *in vitro* pathogenicity testing. The term slime has been used to characterize biofilm formation phenotypically on CRA medium.

Slime production

The isolates were studied for detection of slime production on Congo-red agar medium (CRA). Formation of rough black colonies on CRA plates was considered to be indicative of slime production compared to the red smooth colonies of non-slime producing strains. All the 28 *L. monocytogenes* isolates were streaked on Congo-red agar in order to detect slime production. Out of 28 isolates, 22 (78.57 %) strains of *L. monocytogenes* produced slime. The rest of the 6 (21.43 %) strains were negative for slime production (Table 3). Probably this was the first report on detection of slime in *L. monocytogenes* under Indian conditions. The production of slime, an extracellular substance which surrounds multiple cell layers, facilitates bacterial adherence. Slime production was investigated as a possible major determinant of bacterial adherence to biotic and abiotic surfaces. Slime production in *Staphylococcus*

Table 3: Biofilm and Slime Production of *L. monocytogenes* Isolates

Isolate No.	Slime production	Biofilm production	
		O.D	Remark
LM 1	+	0.287	Moderate
LM 2	+	0.144	Weak
LM 3	+	0.387	Moderate
LM 4	+	0.248	Weak
LM 5	+	0.198	Weak
LM 6	+	0.200	Weak
LM 7	+	0.147	Weak
LM 8	+	0.166	Weak
LM 9	+	0.184	Weak
LM 10	-	0.137	Negative
LM 11	+	0.147	Weak
LM 12	+	0.192	Weak
LM 13	+	0.209	Weak
LM 14	+	0.210	Weak
LM 15	+	0.173	Weak
LM 16	+	0.143	Weak
LM 17	+	0.389	Moderate
LM 18	-	0.127	Negative
LM 19	+	0.378	Moderate
LM 20	-	0.133	Negative
LM 21	+	0.141	Weak
LM 22	+	0.259	Weak
LM 23	+	0.290	Weak
LM 24	+	0.401	Moderate
LM 25	-	0.128	Negative
LM 26	-	0.134	Negative
LM 27	-	0.144	Weak
LM 28	+	0.174	Weak

spp. has been postulated to be associated with their innate resistance to phagocytosis, adhesion, micro colony formation and antibiotic resistance. It has been used as a marker to indicate the ability of the organisms like *Staphylococcus aureus* to adhere the tissues in diseases like mastitis (Vasudevan *et al*., 2003).

Quantification of biofilm

The individual strains of *L. monocytogenes* varied in their biofilm forming ability. Out of total 28, 23 (82.14%) isolates were found to produce biofilm whereas 5 (17.86%) were found negative for biofilm. Biofilm production of *L. monocytogenes* isolates by microtitre plate assay showed O.D. values in the range of 0.128 to 0.438. The cut off O. D. was 0.140. The O.D. value of isolates less than 0.140 was considered negative, while that of 0.141 to 0.280 as weak, 0.281 to 0.560 as moderate and more than 0.560 as strong biofilm producer. The O.D values of all the 28 strains were shown in Table 3. Out of the 28 strains, 5 (17.86 %), 18 (64.29 %), and 5 (17.86 %) were found moderate, weak and negative, respectively, for biofilm production. The strains that produced moderate biofilm belong feces of sheep (2), vaginal swab taken from cattle having reproductive disorder (1), feces of lactating cattle (1), feed (1). Eighteen strains that were classified as weak biofilm producer belong to feces of sheep (5), feces of buffalo (2), vaginal swab taken from buffalo having reproductive disorder (4), vaginal swab taken from cattle having reproductive disorder (2), milk samples of cattle (1) and buffalo (1), while 5 strains that where negative for biofilm production were vaginal swab taken from buffalo having reproductive disorder (1), vaginal swab taken from cattle having reproductive disorder (1), milk samples of cattle (1) and clinical mastitis milk sample taken from cattle (2). Previous study of Doijad *et al*. (2015) also showed that none of the strain from animal clinical cases, human clinical cases, and meat exhibited strong biofilm formation. Biofilm productivity exhibited profound inter-strain variations depending on growth conditions that resulted in inconsistent associations between biofilm phenotype and serotypes throughout the different conditions Lee *et al*. (2019). Further investigations on genes of unknown function as well as a time-course omics approaches such as transcriptomics and proteomics will help decipher the complex mechanisms of biofilm formation

Harveya *et al*. (2007) reported that out 127 of 138 strains (92.0%) were classified as weak, 9 of 138 strains (6.5%) as moderate and only 2 of 138 strains (1.5%) as strong biofilm formers. The strains included environmental, animal, food (persistent and sporadic strains) and clinical isolates. The present findings were in agreement with the above report. Though, the terms slime and biofilm are used interchangeably, the expression of the slime production cannot be correlated with the production of biofilm. As it can be observed in the present study that non-slime producing isolates were also found to be biofilm producers. This finding is in agreement with that of Vasudevan *et al*. (2003) where it was found that three slime negative strains of *Staphylococcus aureus* were also biofilm producers. Therefore, microtiter plate assay can be more consistent assay for biofilm formation in comparison to that of slime production. It has been suggested that bacteria in response to changing environmental conditions were able to switch between a free-living, virulent state and a surface attached, less virulent state. Kalmokoff *et al*.'s (2001) finding that only 1 of 36 clinical *L. monocytogenes* strains formed biofilm on stainless steel surfaces support this suggestion. Bacteria in biofilms were generally more resistant to environmental stresses than their planktonic counterparts. The biofilm forming capability of the *Listeria* spps. makes them particularly successful in colonizing surfaces within the host thus being responsible for persistence infections (Kamelia *et al*., 2016). Furthermore, studies will also be necessary to understand the mechanisms underlying different rates of biofilm growth among strains of *L. monocytogenes*. Knowledge gained in these areas will be an important step towards prevention of biofilms and elimination of persistent strains from food processing environments.

Presence of persistent strains of *L. monocytogenes* in the farm or/and milk line could be due to the residence of mastitic cows and/or a dwelling biofilm in milking machinery and utensils Latorre *et al*., 2013). Biofilms has been linked with bovine mastitis (Melchior et al., 2006a, b). Various organisms of veterinary importance have been successfully grown as biofilms using a Calgary Biofilm Device (CBD) including *Corynebacterium pseudotuberculosis*, *E. coli*, *P. aeruginosa*, *Staphylococcus hyicus* and *S. aureus*. But in present study, it is clear that strains belonging to serotype 4b (predominant serotype responsible for the animal listeriosis and *Listeria*

associated food borne outbreaks and considered to be the most virulent serotype, having proven *in vitro* virulence associated characteristics), showed inconsistent results regarding biofilm production, and none of them was strong biofilm producer. Osman *et al*. (2016) also recorded that 4b strains did not exhibit strong biofilm formation that could have a drastic outcome in the dairy industry with a consequent hazardous implication on food safety. Though epidemiological evidence points to biofilms as a source of several infectious diseases, the exact mechanisms by which biofilm-associated microorganisms elicit disease are poorly understood (Donlan and Costerton, 2002). *L. monocytogenes* biofilm formation is probably controlled by a complex regulation network involved in variable genes required for the different biological pathways. PrfA, a key transcriptional activator that regulates most of the known listerial virulence gene expression, has been shown to promote *L. monocytogenes* biofilm formation. Loss of PrfA dramatically altered gene expression patterns in *L. monocytogenes* biofilm and resulted in reduced ability of the biofilm formation (Luo *et al*., 2013).

CONCLUSION

The present investigation was aimed to assess slime production and quantitation of biofilm production in a set of well-characterized *L. monocytogenes* strains isolated from healthy and clinicaly affected animal sources. Strains belonging to serotype 4b showed inconsistent results regarding biofilm production, and also none of them were strong biofilm producer. Biofilm productivity exhibited profound intra-strain variations irrespective of source of isolation. Biofilm formation is a complex process regulated by diverse factors, and further studies will be necessary to understand the mechanisms underlying the different rates of biofilm production among the strains of *L. monocytogenes*, which will support prevention of biofilms and eliminating persistent strains from animal and animal based food environment. Biofilm formation by such pathogens in the zoonotic pathogen is a matter of concern not only to Veterinarians but also to the human health.

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