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# Antimicrobial Resistance and Biofilm Formation of *Listeria monocytogenes* on Different Surfaces

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**Abstract**

*Listeria monocytogenes* is a major foodborne pathogen capable of forming a biofilm. The objective of this study was to determine biofilm formation by 62 *L. monocytogenes* isolated from ready-to-eat (RTE) foods in Kanchipuram, Tamil Nadu, India. The biofilm formation was determined by the methods like Christenson's test tube method, Congo red agar method, and quantification of biofilm on Catheter assay. In addition, the antimicrobial resistance pattern was analyzed against 12 different antibiotics and compared the antimicrobial resistance pattern before and after biofilm production. Out of 62 isolates, only 6 (9.6%) of isolates were found to be strong biofilm producers, followed by 19 (30.6%) isolates were moderate, 17 (27.4%) isolates were weak biofilm producers, and 20 (32.2%) isolates were non-biofilm producers. Based on the result of the tube test method positive biofilm-formed isolates were subjected to Congo red agar plates. Among 42 isolates tested, it was found that 42 (85.7%) isolates formed biofilm. The randomly selected six biofilm-producing *L. monocytogenes* strains were subjected to biofilm formation on some catheters and the results revealed positive adherence of *L. monocytogenes.* The antimicrobial resistant pattern was high after biofilm formation. This study exposed that *L. monocytogenes* have formed biofilm and this condition leads to a serious threat to humans.

**Key words :** Foodborne pathogen, *Listeria monocytogenes*, Ready to eat food, antimicrobial resistance, Biofilm, Catheter.

***L. monocytogenes*** are gram-positive, facultatively anaerobic, non-spore-forming, motile at 22-28°C but non-motile at 30°C, rod-shaped bacterium. The optimum temperature for growing at 37°C but *L. monocytogenes* is able to grow at the range

from -0.4°C to 45°C temperature[15]. *Listeria monocytogenes* is a major, ubiquitous, foodborne pathogen that can contaminate food products manufacturing or food processing. *Listeria monocytogenes* cause listeriosis. RTE foods are one of the sources of *L. monocytogenes* spreading to human beings. *L. monocytogenes* poses an important risk to the food industry and mostly affects producers of ready-to-eat (RTE) foods. Due to its capability to increase over an enormous range of adverse conditions about low temperature, low pH, and high salt. *L. monocytogenes* represents a major public health issue for it may cause severe human sickness with serious consequences[14]. Biofilm formation is the characteristic phenomenon of the microorganism. The highest number of bacteria adhere to the sluggish surface of the cell through extracellular polymeric matters is consequent as biofilm[9]. A biofilm produced by bacteria is naturally protected from harmful environmental factors, for example, acids or alkalis, antibiotics, osmotic stress, and UV radiation[4]. When developed biofilm, the self-produced extracellular polymeric matrix gives extra protection to bacteria from harsh environmental conditions such as nutrient deprivation, desiccation, or disinfectant treatment[8]. Bacterial biofilms can attach to various materials such as glass surfaces, metals, plastic wares, clinical devices, and tissues. Bacterial communities also produce biofilm, mainly on all medical implants, including catheters, heart valves, intrauterine devices, pacemakers, vascular grafts, prosthetic joints, sutures, and contact lenses for acute infections[10]. As a consequence, it is challenging to control bacterial contaminations. Despite an initial belief that *L. monocytogenes* might only form monolayer biofilms, future studies demonstrated a variable degree of biofilm maturity[12].

In addition, some infectious microorganisms are maybe related to biofilm pathogens and cause risky infections in humans. It is a big challenging incidence in the medical field. Biofilms are frequently associated with an augmented risk of catheter-related infection. Urinary tract infection (UTI) is the most general, nosocomial infection, and its main cause is the urinary catheter, which is an implanted medical device. A number of patients hospitalized inserted urinary catheters at least once during their stay, and operations, who are in intensive care units[11]. In the strict measures that have been taken to prevent hospital-associated UTI, biofilm producers have been a great obstacle in terms of effective treatment and cure. Females are highly weak to this infection than males. They happen regularly from the ages 16 to 35 years, with more than 10% of females affected by an infection in their life period. Also, the reappearance of this infection is normal, and predictable 50% of people suffer due to reinfection within a year[1].

Furthermore, the bacterium that has the notable property of biofilm formation showed higher antimicrobial resistance compared to the planktonic forms and it can be revealed as 1000 times more resistant to antibiotics related to normal pathogens[13]. Sometimes, opportunistic environmental circumstances may favour biofilm-producing bacteria done more virulence molecules and become resistant to effects[18]. The formation of biofilms in medical devices is an increasing concern due to the observed antimicrobial resistance of colonizing microorganisms.

Currently, some pathogens are already known for their multi-resistance to antimicrobials. To overcome this problem, new strategies are being developed that prioritize the prevention of biofilm formation, such as device surface modification or coating[5].

A comprehensive study of biofilm helps the development of procedures to withstand biofilm formation. Several studies reveal the connection between biofilm formation and antimicrobial resistance. Investigates on biofilm formation on several surfaces are still not adequate. Therefore, this study aims to assess antimicrobial susceptibility and biofilm formation of *L. monocytogenes* on different surfaces.

*Bacterial isolates :*

In this study, 62 *Listeria monocytogenes* were isolated from RTE foods in Kanchipuram, India, and identified by a biochemical test, CAMP test, and 16s rRNA sequencing.

*Biofilm formation assays :*

*Christenson's test tube methods :*

All the *L. monocytogenes* overnight cultures were inoculated into 10 ml BHI broth and incubated in the test tubes without shaking for 24 h at 37°C. After the incubation period, the tubes were drafted out and washed with PBS, and dried. Following, the tubes were stained with crystal violet stain (0.1%), and the tubes were washed with distilled water to remove excess stain. Then, all the tubes were examined and the result of biofilm formation was recorded as weak, moderate, strong, and absent[16].

*Congo red agar method :*

The isolated biofilm-producing bacterial strains based on the tube test method were subjected to one more biofilm formation detection method like culturing on Congo red agar (CRA) plates. Agar plates were prepared by adding 0.8g Congo red stain (Himedia) and sucrose (Himedia). Then, the CRA plates were inoculated with *L. monocytogenes* strains and incubated at 37°C for 48 h. The plates observed black color colony formation indicating positive and pink colors resembled negative biofilm formation[3].

*Biofilm formation in catheters :*

The biofilm-forming capability of the *L. monocytogenes* was further studied, by determining the actual number of cells present in the biofilm. Six representative strains from each kind of biofilm producer (weak, moderate, and strong) were selected for the study to differentiate the biofilm-forming ability. Biofilm forming capability on the catheter was quantified using an improved crystal violet assay. A 13mm lengthy piece of two types of catheters (latex and silicon–coated) was cut vertically and placed at the bottom of a test tube containing Luria - Bertani (LB), sterile artificial urine (1% NaCl, 2.43% Urea, 0.6% KCL, 0.64% $Na_2HPO_4$, 0.05 mg/ml albumin, pH 5 - 7), and natural urine. Then, 0.1ml overnight culture was transferred in all tubes and incubated at 37°C for 24 h. After 24 h, unbound cells were washed with water. The piece of the catheter was transferred to a fresh tube to avoid the estimation of biofilm formed at the base of the earlier tube. Bound biofilm was fixed with 5ml methanol and stained with

5ml of 0.5% crystal violet. The excess stain was washed with 0.8% saline, the bound stain was resolubilized in 5ml of 33% acetic acid and its OD was measured at 570 nm. Uninoculated LB, artificial sterile urine, and natural urine were used as negative control[6].

*Antimicrobial susceptibility :*

The antimicrobial-resistant pattern was examined in all the isolates before and after biofilm production by using the disk diffusion method on Muller – Hinton agar (Himedia). The *L. monocytogenes* was tested against 12 different antibiotic disks (Himedia). The antibiotic discs used were Penicillin G - P (10 unit), Amoxicillin - AMX (10 mcg), Carbenicillin - CB (100 mcg), Methicillin - MET (5 mcg), Azithromycin - AZM (15 mcg), Clindamycin -CD (2 mcg), Lincomycin -L (2), Vancomycin - VA (30 mcg), Rifampicin -RIF (5 mcg), Roxithromycin - RO (15 mcg), Teicoplanin - TEI (30 mcg), and Linezolid - LZ (30 mc9g). The result was interpreted as resistant, intermediate, or susceptible as described by the CLSI guideline[17].

*Christenson's test tube methods :*

The result of biofilm formation in the tube method was evaluated by using a 0.1% Crystal Violet stain. Biofilm formation was considered positive when a visible purple color-lined was formed on the wall and bottom of the tube. Among the isolates, only 6 (9.68%) isolates showed a strong form of biofilm on the glass surface, followed by 19(30.64%) isolates that were found to be moderate biofilm producers, and 17(27.41%) isolates showed weak biofilm on the glass tubes. However, the remaining 20 isolates (32.26%) were not showed purple color on glass tubes, that consider non-biofilm producers (Table -1).

Table – 1. Biofilm forming capability of the 62 *L. monocytogenes* from RTE foods using tube test Method:

| S. No. | Types of biofilm producers | Total number of biofilm producers | Percen-tage (%) |
|---|---|---|---|
| 1 | Strong | 6 | 9.67% |
| 2 | Moderate | 19 | 30.64% |
| 3 | Weak | 17 | 27.41% |
| 4 | Non–biofilm producer | 20 | 32.25% |

*Congo red agar method :*

The formation of biofilm in the Congo red agar method exhibited high biofilm production than in the test tube method. Of a total of 42 strains that showed positive in the test tube method, 36(85.71%) isolates of *L. monocytogenes* were found to be biofilm producers whereas 6(14.28%) isolates did not produce (Table-2).

Table – 2. Congo red agar method:

| S. No. | Types of biofilm producers | Total number of biofilm producers | Percen-tage (%) |
|---|---|---|---|
| 1 | Biofilm producer | 36 | 85.71% |
| 2 | Non-biofilm producer | 6 | 14.28% |

*Biofilm formation in catheters :*

Biofilm formation by randomly selecting 6 isolates on two different catheters

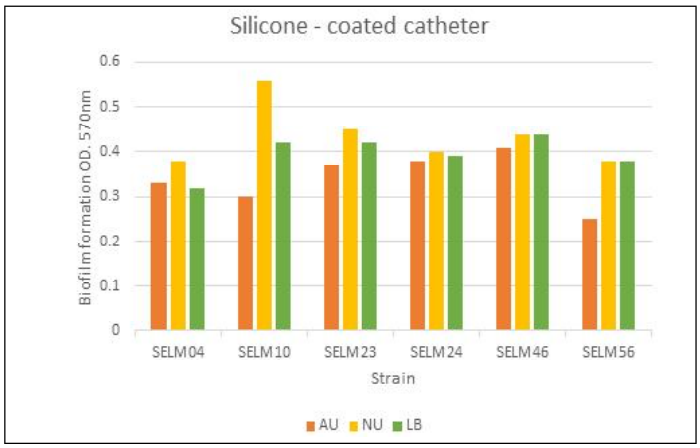


Figure 1. Biofilm Formation in Silicone Coated Latex Catheter:
(AU – Artificial urine, NU- Natural urine, LB- Luria -Bertani broth).

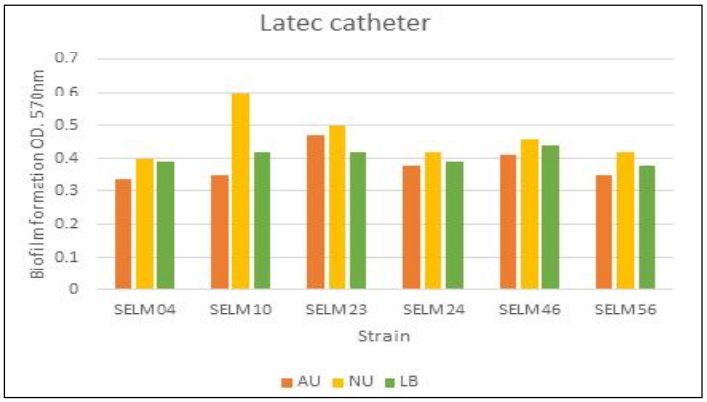


Figure 2. Biofilm Formation in Latex Catheter:
(AU – Artificial urine, NU- Natural urine, LB- Luria -Bertani broth).

in three different media was examined and the results are depicted in Figures 1 and 2. The results revealed that biofilm formation in all six strains of *L. monocytogenes* was higher when both catheters were placed in natural urine (NU) followed by Luria - Bertani broth (LB) and artificial urine (AU). In general, it was found that latex catheters provided a conducive environment to enhance biofilm formation than silicone-coated catheters. Among the strains tested, strain number SELM10 exhibited high biofilm on both catheters (Figures 1 and 2).

*Antimicrobial resistance pattern of L. monocytogenes before and after biofilm formation :*

The disk diffusion evaluation of the 62 isolates of both before and after biofilm formation of *L. monocytogenes* from RTE

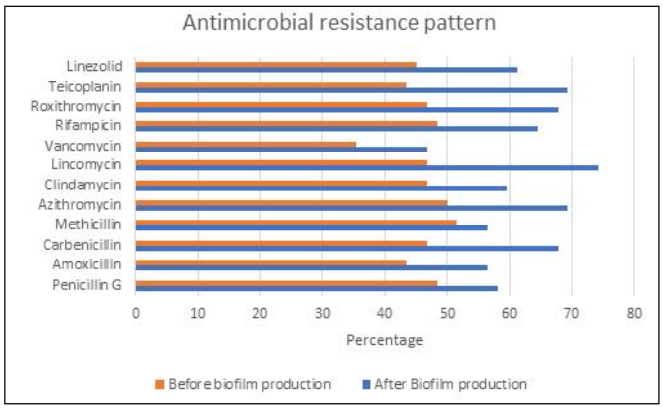


Figure 3. Antimicrobial resistance pattern of *L. monocytogenes* before and after biofilm formation.

foods is clearly depicted in Figure 3. From the result, before biofilm formation, *L. monocytogenes* revealed maximum resistance against Methicillin (51.61%), followed by Azithromycin (50%), Penicillin G, and Rifampicin (48.38%), Carbenicillin, Clindamycin, Lincomycin, and Roxithromycin (46.77%), Linezolid (45.16%), Amoxicillin and Teicoplanin (43.54%) and Vancomycin (35.48%). Interestingly, however, after biofilm formation, it was found that *L. monocytogenes* exhibited maximum resistance against Lincomycin (74.19%), followed by Azithromycin and Teicoplanin (69.35%), Carbenicillin and Roxithromycin (67.74%), Rifampicin (64.51%), Linezolid (61.29%), Clindamycin (59.67%), Penicillin G (58.06%), Methicillin and Amoxicillin (56.45%), and Vancomycin (46.77%).

In recent years, the consumption of RTE foods has augmented; accordingly, there have been *L. monocytogenes* spreading in foods such as mushrooms, packaged salads, and frozen vegetables. In addition, *L. monocytogenes* can develop biofilm in a food processing environment and medical devices. Sixty percent of epidemics are caused by biofilm-related infections through pathogens and antimicrobial resistance, which have an important impact happening the food industry[3].

In the present study, 62 isolates were examined for biofilm production by Christenson's test tube methods. Among these, 6 isolates were strong biofilm producers, 19 isolates were moderate biofilm producers, 17 isolates were weak biofilm producers, and 20 isolates were non-biofilm in nature. Our findings are comparable with the reports of Osman *et al.*,[16] who previously reported biofilm-producing *L. monocytogenes* by Christensen's tube method. The isolates were phenotypically categorized by Congo red agar plates and the results were lower when compared to the report of Novoa *et al.*,[3] who phenotypically characterized biofilm-producing *L. monocytogenes* by Congo red agar plates.

The biofilm-formation capability of selected strains of *L. monocytogenes* has quantified the catheter (latex and silicone coated). In the presence of natural urine and artificial urine along with Luria - Bertani broth. In general, biofilm formation was higher in latex

catheters in all the media than in catheters coated with silicon. This is in agreement with the results of Desai *et al*.,[6] who previously reported on *Klebsiella pneumonia* through catheter assay. Silicon in the catheter could have slowed down biofilm in the catheter and this needs further investigation.

Biofilm formation was high in natural urine, LB broth, and artificial urine. The latex is less cytotoxicity and might be enhanced biofilm formation. Therefore, in this study, the latex catheters were coated with silicone elastomer and found to decrease biofilm. Most modern catheters are made completely of silicone and hydrophilic coatings, which are used to offer a slippery surface to decrease attachment. Silicone catheters are not only hypoallergenic, but they also have exposed reduced biofilm formation associated with latex. Donlan.,[7]. As previously reported by Lee *et al*.,[11] the rough surface of latex catheters makes bacterial attachment easy and an extra amount of biofilm formation occurs, whereas the smooth surface and less hydrophobicity of silicone catheters are responsible for reduced biofilm formation. Our findings corroborate these findings courtesy of silicone catheters being preferred over latex concerning biofilm formation.

Further, the antimicrobial susceptibility was compared with the cells from biofilm producers and non-biofilm producers. The cells biofilm producers showed increased resistance when compared to non-biofilm producers. Atray and Atray.,[2] previously reported a correlation between biofilm-produced and non-biofilm-produced *E. coli* and their antibiotics resistance patterns and these findings were higher than our findings. Therefore, the resistance values were higher in biofilm producers when compared to non-biofilm producers. The biofilm capability and the increased antimicrobial resistance of *L. monocytogenes* and other pathogens is a serious concern in view of public health.

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**Conflict of interest :**

The authors have no conflict of interest.

**Author's contribution :**

SE collection of literature review sources, investigation, designing the tables and figures, and writing the original draft. BR, KPK, and KS did the Conceptualization, Methodology, Writing review & Editing, Supervision, and Project Administration.

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