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Crystal Violet Binding Assay for Assessment of Biofilm Formation by *Listeria monocytogenes* and *Listeria* spp on Wood, Steel and Glass Surfaces

Victoria O. Adetunji and Tajudeen O. Isola

Department of Veterinary, Public Health and Preventive Medicine, University of Ibadan, Ibadan, Nigeria

Abstract: Micro-organisms attach to surfaces and develop biofilms which are a concern in food and environmental safety. This study, quantified biofilms in 20 isolates viz: *Listeria monocytogenes* (14) and *Listeria spp* (6) from meat tables in Ibadan municipal abattoir, Nigeria on wood, glass and steel surfaces using crystal-violet binding assay. *Listeria* isolates formed biofilms on all three surfaces. At 24hours *Listeria monocytogenes*(SLM) formed more biofilms on wood(0.22±0.01) and glass(0.09±0.01) surfaces than *Listeria spp*(SLS) which was wood(0.17±0.02) and glass (0.06±0.01). But the reverse was the case on steel where *Listeria spp* (SLS) formed more biofilms (0.11±0.01) than *Listeria monocytogenes* (SLM) (0.10±0.00). A significant difference ($P<0.01$) exists between isolates for wood and glass at 24hours and ($P<0.05$) for wood at 72hours. Significant differences ($P<0.05$) occured in biofilms produced between 24hours and 72hours. It can be concluded that selection of suitable surfaces could reduce the ability of bacteria forming biofilms on meat contact-surfaces. This finding is very relevant in developing countries where wood is still in use as a major meat contact-surface in abattoirs.

Key words: Biofilm · *Listeria* spp · Abattoir · Meat · Contact-surfaces

INTRODUCTION

Nearly 99% of micro-organisms living on the earth live in microbial communities known as biofilms [1]. Biofilms are formed by adhesion of bacterial cells to surfaces through an exopolymeric matrix. This matrix is important in formation and structure of the biofilm and also on the protection of the bacterial cells as it prevents antimicrobials and xenobiotics from gaining access to the cells inside the biofilm [2]. Biofilm formation can compromise the cleanliness of food contact surfaces and environmental surfaces by spreading detached organisms to food and other areas of processing plants [3]. Biofilms formed in food processing environments are important as they have the potential to act as a chronic source of microbial contamination that eventually leads to food spoilage and transmission of diseases [1, 4]. Biofilms lead to serious hygienic problems and hence, the important aspect of controlling biofilms represents one of the most persistent challenges within food and industrial environments where microbial communities are problematic [5]. Biofilm forming bacteria persist and produce after sanitation thus making biofilms a potential threat to food safety [6].

Various methods have been used for biofilm assessment; one of these is the crystal violet binding assay as described by Stepanovic and others [4, 22]. In this assay, the dye bound to adherent cells is resolubilized and measured in optical density (OD).

Listeria spp are gram positive bacilli and are typified by *Listeria monocytogenes*, the causative agent of listeriosis. Clinical infections are primarily by *Listeria monocytogenes although Listeria ivanovii* is also pathogenic being particularly associated with abortion in ruminants. The remaining species are recognized as non pathogenic. *Listeria monocytogenes is* quite hardy and resists dexterous effects of freezing, drying and heat remarkably well for a bacterium that does not form spore [7].

Listeria's tolerance has attributed to it being isolated from various meat samples, with chicken being the best host for growth of the pathogen [8]. Listeriosis is contracted through the consumption of contaminated foods [9]. *Listeria monocytogenes* is found in a variety of food products such as soft cheeses, dairy products raw foods, ready to eat products and equipment surfaces [10, 11]. *Listeria monocytogenes* is a food borne pathogen that

Corresponding Author: Victoria O. Adetunji, Department of Veterinary, Public Health and Preventive Medicine, University of Ibadan, Ibadan, Nigeria, E-mail: vadetunji@gmail.com & vo.adetunji@mail.ui.edu.ng.

account for less than 1% of food borne illness but is responsible for 28% of deaths caused by food borne diseases [12].

Several studies have been conducted to determine the ability of *Listeria monocytogenes* to adhere and form biofilms on food contact surfaces. All of which determined that *L. monocytogenes* attach to food industry surfaces such as plastics, glass, stainless steel and rubber surfaces [13-20]. However, it has been noted that there are differences in both the extent and the rate of adsorption. Luden *et al [*21] demonstrated that the most prevalent strain of *L. monocytogenes* (strain $1/_2$c) found in food processing plants has good adhesion ability and required only a short contact time for attachment.

This work presents an assessment of biofilms formed by isolates of *Listeria monocytogenes* and *Listeria spp* from a tropical abattoir on wood, glass and steel surfaces using the crystal violet binding assay.

MATERIALS AND METHODS

Preparation of *Listeria* Isolates Culture for Attachment and Biofilm: *Listeria monocytogenes* isolates (SLM 14) and *Listeria spp* (SLS 6) were isolated from meat tables in Ibadan municipal abattoir, Nigeria. Modified oxford agar base (MOX) for Listeria isolation was supplemented with antibiotic supplements (acriflavine, nalidixic acid and cycloheximide) (Becton, Dickinson and company) was used for Listeria isolation. Further identification of the isolates was based on procedures described by Barrow and Feltham [22]. One colony of each *Listeria* isolates was cultured on nutrient agar slope (Fluka 7014, Germany) and was transferred into each of the 20 sterile glass jars with lids containing 150ml of nutrient broth (Fluka 7014, Germany). One glass jar not inoculated with *Listeria* isolate served as control in this study. The inoculated broth culture was incubated at 37°C for 24hours. Two hundred and seventy-six (276) chips (90 each (4cm x 2cm x 1cm) for glass (Easy way medical, England), steel (type 304, #4, Ajaokuta steel co., Nigeria) and wood (softwood, Oak) were used. The chips were washed with detergent (Unilever, Lagos, Nigeria), rinsed with sterile distilled water and air-dried before being placed into hot air oven (Elektro-Helios, Sweden) at 75°C for 30mins. After 24hours incubation of the inoculated broth culture, four of steel, glass and wood chips were aseptically put into each of the glass jars making a total of 12 chips per glass jar. All the glass jars were incubated at ambient temperature of (26-28°C) for 24 hours and 72 hours. At the end of each incubation period, a set of chips were aseptically removed from the broth culture for biofilm quantification using the crystal violet binding assay described by Stepanovic *et al.* [4]. Each set of chips was washed 3 times with 5ml of sterile distilled water. The remaining adhered bacteria were fixed with 2.5ml of methanol per chip. Each chip was stained with crystal violet (Fishers scientific, USA) for 15 minutes and then the excess stain washed off under running tap water. After the chip was air dried, the dye bound to the adherent cells was re- solubilized with 2.5ml of 33% glacial acetic acid (Fishers scientific, USA) for each chip. The re-solubilized liquid for each chip was poured into a cuvette. The absorbance (optical density) of each re-solubilized liquid was measured against the optical density of blank reading without inoculation (control) at wavelength of 620nm for the Listeria *strains* using a spectrophotometer (Springfield, UK). The absorbance of negative control was subtracted from the absorbance values to determine the actual values [23].

Statistical Analysis: All results were presented in mean±standard error of mean (Mean±SEM). A one-way analysis of variance (ANOVA) was used to determine significant differences between means for each surface and strains. Student t- test was used to assess significant differences between strains. All data were analyzed using SPSS 15 [24], Chicago and IL., USA. Statistical significance was evaluated at $P<0.05$ and $P<0.01$. Charts were plotted using Microsoft Excel [25].

RESULTS

The mean absorbance values at 24hours showed that *Listeria monocytogenes* (SLM) (Figure 1) formed more biofilms on wood (0.22±0.01) and glass (0.09±0.01) surfaces than Listeria *spp* (SLS) which was wood (0.17±0.02) and glass (0.06±0.01). But the reverse was the case on steel where *Listeria spp* (SLS) formed more biofilms (0.11±0.01) than *Listeria monocytogenes* (SLM) (0.10±0.00) respectively (Table 1). The mean absorbance value at 72hours showed that *Listeria monocytogenes* (SLM) formed more biofilms on wood (0.35±0.01) and glass (0.12±0.01) surfaces than *Listeria spp* (SLS) which was wood (0.28±0.01) and glass (0.11±0.01) respectively (Table 1). The mean absorbance values (nm) of biofilm adherence to surfaces were observed at 24 and 72hours on wood more significantly ($P<0.05$) than on glass and steel surfaces for the isolates except *Listeria spp* at 24hours where there were significant differences ($P<0.05$) in biofilm formation on the three surfaces (Table 1).

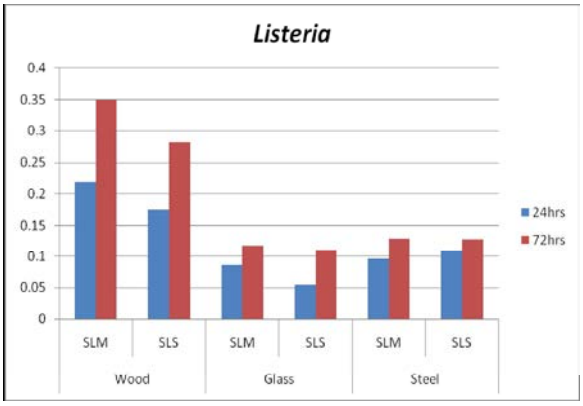

Fig. 1: Showing differences in biofilms produced by SLM-*Listeria monocytogenes and* SLS-*Listeria spp* on the 3 surfaces at 24 and 72hours

Table 1: Comparison of *Listeria* biofilms on wood, steel and glass surfaces at 24 and 72 hours incubation period

		SURFACES		
Strains (Codes)	Periods	Wood (mean absorbance (nm)	Steel(mean absorbance (nm)	Glass (mean absorbance (nm)
SLM	24hrs	0.22±0.01[a]	0.10±0.00[b]	0.09±0.01[b]
SLS	24hrs	0.17±0.02[a]	0.11±0.01[b]	0.06±0.01[c]
SLM	72hrs	0.35±0.02[a]	0.13±0.01[b]	0.12±0.01[b]
SLS	72hrs	0.28±0.03[a]	0.13±0.01[b]	0.11±0.00[b]

* *Means with the same superscript are not significantly different at 0.05 levels

DISCUSSION

In this study, biofilm formation by *Listeria monocytogenes* and *Listeria spp* revealed that these bacteria possess a high capacity for biofilm formation on the 3 surfaces used (wood, glass and steel) but with differences in the extent of adhesion. Previous studies have confirmed the biofilm formation by strains of *Listeria monocytogenes* and *Listeria spp* on these surfaces [13, 14, 19, 20, 26]. The higher level of biofilm production by isolates of *Listeria monocytogenes* when compared to *Listeria spp* on wood and glass at 24hours has been explained in other studies done by Hood and Zottola [13]; Gulsun *et al*. [27] where they reported that the level of biofilm production of isolates is a virulence characteristic of such isolates. In the current work, the mean absorbance values (nm) of biofilms produced by *Listeria* showed that wood retained the greatest biofilms followed by steel and glass. This result is in agreement with the report of Sinde and Carballo, [19]; Donlan [28] who reported that glass and stainless steel are hydrophilic materials while wood and plastic are hydrophobic materials. Hydrophobic materials are reported as surfaces that provide a greater bacterial adherence [29]. Fletcher and Loeb [30] noted that large numbers of bacteria attached to hydrophobic surfaces with little or no surface charge and moderate numbers attached to hydrophobic metals with positive charge or neutral charge and very few attached to hydrophilic negatively charged substrates. The importance of hydrophobicity in bacteria attachment has also been highlighted by other investigators [31]. Variation in biofilm density depending on surface was also reported by Karunasagar *et al*. [32] with *Vibrio harveyi*. Cunliffe *et al*. [33]; Sinde and Carballo [19]; Donlan [34] also reported that *Listeria monocytogenes* adhere in higher numbers to more hydrophobic materials. Donlan [34] also reported that adhesion is the first step in the complex process of biofilm formation.

The finding of significant differences ($P < 0.05$) in biofilm formation between 24 and 72 hours incubation periods for all the *Listeria* strains (Figure 1) in this study has also been demonstrated by Moltz and Martin; [35]; Adetunji [36]; Adetunji and Adegoke [37] on stainless steel chips and quantitative recovery of purified biofilm and cellulose from culture respectively. In these studies increase in biofilms formation with extension of incubation period was reported.

Use of wood as a food contact surface has reduced in the developed countries because it is a porous and absorbent material where organic matter along with bacteria can become entrapped; cross-contamination is a main concern [38] but wood is still in use in the developing countries for food processing because it's readily available and cheap. The preferred surface for food processing is glass. Glass is sometimes used for food contact surfaces because of its smooth and corrosion-resistant surface [39]. On the other hand, stainless steel resists impact damage better than glass but is vulnerable to corrosion, while rubber surfaces are prone to deterioration and may develop surface cracks where bacteria can accumulate [40]. Equally important in meat contact surface is the cleanability of the surface once bacteria forming biofilm have attached. The use of wood tables for meat display should be discouraged in the developing countries based on the findings in this study.

CONCLUSION

It is concluded that *Listeria monocytogenes* and *Listeria spp* isolates from meat tables in the abattoir under study are capable of forming biofilms on wood,

steel and glass surfaces but with some variations. The food processing units in the developing countries should therefore consider the application of findings in this study with a view to preventing biofilms in food processing environment. Further studies will be necessary to understand whether the ability to form biofilms may influence the survival of strains in meat contact surfaces.

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