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A Procedure to Test Biofilm Formation Capacities of Listeria Monocytogenes Strains Under Conditions Simulating Natural Meat Processing Conditions

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We use this protocol and it's working

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Abstract

Biofilm formation is believed to be one of the mechanisms that enables *Listeria monocytogenes*, a food-borne bacterial pathogen, to persist in food processing plants. It is important to determine the differential abilities of biofilm formation of various *L. monocytogenes* strains under natural conditions for gaining insights into this mechanism and for developing effective mitigation strategies. The protocol presented here outlines an in vitro biofilm assay developed in our laboratory to detect biofilm formation in *L. monocytogenes* strains. This assay uses a standard crystal violet biofilm assay in a novel model (in-house prepared Beef Broth, 12ºC), closely simulating natural meat processing environments. The biofilm formation is measured using optical density values and confirmed morphologically using inverted microscopy and scanning electron microscopy.

Guidelines

Background

Listeria monocytogenes, a Gram-positive food-borne pathogen, can cause a serious infection in humans called listeriosis. Individuals in vulnerable groups, such as newborns, the elderly, pregnant women, and the immunocompromised, are particularly at higher risk. The pathogen has been shown to persist in harsh food processing environments, posing a significant threat to public health. This persistence is primarily attributed to its ability to form biofilms. Therefore, understanding the biofilm-forming ability of *L. monocytogenes* by testing strains under simulated meat processing conditions is crucial, as it provides insight into its persistence in these challenging environments. Previously, we conducted a study to test the biofilm formation capacities of 67 *L. monocytogenes* strains and obtained biofilm OD_{595} values (optical density values at 595 nm) that ranged from OD_{595} 0.076±0.035 to 0.517±0.027.

Purpose

This protocol is to describe a biofilm bioassay simulating the food processing environment to measure biofilm formation of *Listeria monocytogenes* in beef broth at 12ºC following 9 days of incubation (Soosai D, 2017).

Trouble shooting

1. Check the sterility of the in-house prepared beef broth by incubating the broth and 2 BHI agar plates streaked with the broth at 37 °C Overnight .
2. Include negative and positive controls for biofilm bioassay to ensure there is no cross contamination.
3. Wash the tissue culture plates gently without disturbing the biofilms in the well.
4. Slightly variations in counts between wells of the same strain may be observed. To obtain more accurate results, it is recommended to include more than 3 replicates.

Materials

Bacterial strains

All isolates of *L. monocytogenes* are used immediately after isolation or stored in Brain and Heart Infusion (BHI) broth containing 25% (v/v) sterile glycerol at –80 ºC until use.

Reagents

- Glycerol (Catalogue# G5150, Sigma, Mississauga, Ontario, Canada or equivalent), Sterilized by autoclaving at 121 ºC.
- TSB supplemented with 0.6% (w/v) yeast extract (YE) (Catalogue# LP0021, OXOID, Nepean, Ontario, Canada) [TSB-YE].
- In-house Beef Broth (BB) 0.1% (v/v) (Section: Preparation of 1% (v/v) beef broth (BB) in sterile MQ water).
- Phosphate buffered saline (PBS, 0.01M, pH 7.2).
- Brain Heart Infusion (BHI) agar plates.
- Sterile Milli-Q (MQ) water (autoclaved).
- Gram's crystal violet (Cat# 4312526, BD, through Fisher Scientific, Nepean, Ontario, Canada).
- 95% (v/v) ethanol (de-staining solution).
- 2.5% glutaraldehyde (EM Grade 70% solution, Cat. #16365, Electron Microscopy Sciences)
- 2% osmium tetroxide (Cat. #19134, Electron Microscopy Sciences)
- Silver conductive adhesive (Cat. #12684-15, Electron Microscopy Sciences)

Equipments

- 24 well non-treated tissue culture plates (Cat # 08772-51, Falcon, through Fisher Scientific, Nepean, Ontario, Canada).
- Stomacher 400 circulator, Seward, Worthing, West Sussex, U.K, with Stomacher® closure bag (Cat# BA6141/CLR, Seward), Stomacher® filter bag (Cat# BA6141/STR, Seward).
- Allegra X-15R Centrifuge, Beckman Coulter, Brea, California, USA.
- Titer plate shaker (Model # 4625, Lab - Line Instrument Inc., Melrose Park, Illinois, USA).
- Microplate reader (Cat # 168-1135, Bio-Rad i-Mark Microplate Reader, Hercules, California, USA).
- UV-Vis Spectrophotometer (Genesys 10S UV-Vis Spectrophotometer, Thermo Scientific).
- A vacuum hand operator for aspiration (Integra Biosciences™ Vacuboy Hand Operator, Thermo Fisher Scientific).
- Inverted microscope (option: Zeiss Axiovert 10 inverted microscope (Oberkochen, Germany).
- Scanning Electron Microscopy (optional).
- cell culture microscope slides (Cat. #71888, Electron Microscopy Sciences, Hatfield, Pennsylvania, USA)
- 12.7 mm slotted stubs (Cat. #75210, Electron Microscopy Sciences)
- 12 mm Pelco™ carbon conductive tabs (Cat. #16084-1, Ted Pella Inc., Redding, California, USA)

Glycerol **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**G5150

- Oxoid™ Yeast Extract Powder, 500g **Thermo Fisher Catalog #**LP0021B
- BD Gram Crystal Violet **Fisher Scientific Catalog #**B4312526
- Falcon™ Polystyrene Microplates **Fisher Scientific Catalog #**08-772-51
- Stomacher® 400 Circulator Standard Bag **Seward Catalog #**BA6141/5
- PELCO Tabs™, Carbon Conductive Tabs, 12mm OD **Ted Pella Inc. Catalog #**16084-1
- Aqueous Glutaraldehyde EM Grade 70% **Electron Microscopy Sciences Catalog #**16365
- Osmium Tetroxide **Electron Microscopy Sciences Catalog #**19134
- Silver Conductive 18Db70X **Electron Microscopy Sciences Catalog #**12684-15
- AIM Mount Slotted Head **Electron Microscopy Sciences Catalog #**75210

Preparation of 1% (v/v) Beef Broth (BB) in Sterile MQ Water

1h 12m

1 Commercial medium ground beef (approximately 23% fat) is purchased from a retail store.

2 Add beef to sterile MQ water in the ratio of 1:10 (w/v) in a Stomacher® closure bag.

3 Stomach at 300 rpm, 00:02:00 . 2m

4 Centrifuge beef suspension at 230 x g, 00:10:00 to spin down fat and cellular debris. 10m

5 Add the suspension to a Stomacher® filter bag to remove the debris and collect the clear suspension from the filtered side of the bag.

6 Heat the suspension at 72 °C in a water bath for 01:00:00 . 1h

7 Aseptically aliquot the BB into 15 ml centrifuge tubes and store at -20 °C until use.

In Vitro Biofilm Assay

23h

8 Culture isolates at 37 °C Overnight in 10-12 mL of (Tryptic Soya Broth (TSB) supplemented with 0.6% (w/v) yeast extract (YE) (TSB-YE) by inoculating 1/8th of a 10 µL loop from Brain Heart Infusion (BHI) agar plates and incubate for 22:00:00 . 22h

9 Dilute overnight culture in phosphate buffered saline (PBS, [M] 0.01 Mass Percent , pH pH 7.2) to the required OD_{600} value.

Note

OD_{600} 0.180 – 0.230 which is approximately equivalent to 10^8 CFU/ml.

10 Dilute the culture suspension further to obtain a final concentration of 10^6 Colony forming unit per milliliter (CFU/ml) in BB.

11 Confirm the concentration of the diluted bacterial culture to be 10^6 CFU/ml by plating on BHI agar plates for each experiment.

12 Seed the wells of a 24 well culture plate with 2 mL of the bacterial suspension.

13 Use wells containing media alone as a negative control.

14 Use bacterial suspension from one strain identified as a strong biofilm former, either through literature or in-house testing, as the positive control.

Note

In our case, the overnight culture of the positive control strains is standardized to an OD_{600} 0.120 – 0.130 (~10^7 CFU/ml) before performing the final dilution (~10^6 CFU/ml) for biofilm assay.

15 Incubate plates with lids on at 12 °C for 9 days.

16 Remove the culture supernatants gently either manually or by aspiration using a vacuum-based hand operator.

17 Rinse the plates three times with approximately 4 mL of water and allow to air dry.

18 Stain the biofilms by adding 2 mL of 0.1% (v/v) Gram's crystal violet and incubate at ambient temperature for 00:30:00 . 30m

19 Remove the dye and rinse the wells three times with MQ water.

20 De-stain the bound crystal violet by adding 0.5 mL of 95% (v/v) ethanol (de-staining solution) to the wells with shaking on a titer plate shaker for 00:30:00 . 30m

21 Confirm or verify the potential biofilm formation, if necessary, daily, or periodically by visualizing the crystal violet-stained biofilms in the wells of the 24 well plate for the controls and test strains using inverted light microscopy at x20 magnification. Figure 1 (as an example) and/or using scanning electron microscopy (image not shown).

Figure 1. Confirmation of the biofilm formation by inverted light microscopy.

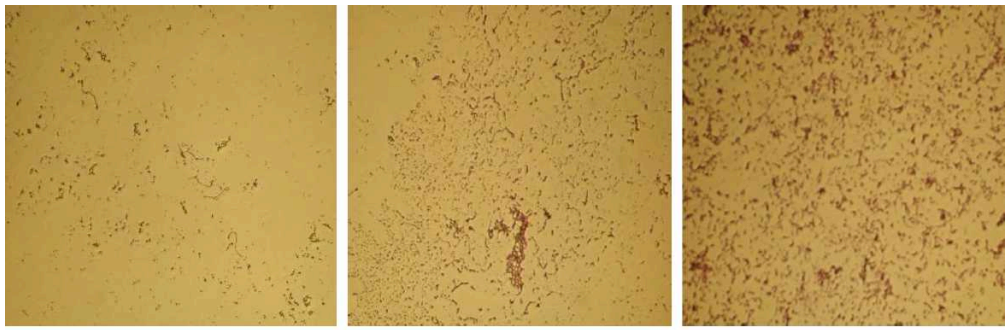

Figure 1: Inverted light microscopy images of *L. monocytogenes* biofilm formation. Images taken at a magnification of ×20 show clear differences in the formation of biofilms stained using crystal violet on days 1, 4 and 9 (the strongest formation) at 12 ºC in BB.

22 Transfer 200 µL of the dissolved crystal violet in the de-staining solution to wells of a 96 well plate and read using a microplate reader at 595 nm.

Morphological Confirmation of Biofilm Formation by Inverted Microscopy

23 View crystal violet stained biofilms in the wells of the 24 well plate using an inverted microscope* at x20 magnification every day to verify potential formation of biofilms (Figure 1).

Note

***Optional**: Zeiss Axiovert 10 inverted microscope, Oberkochen, Germany.

Morphological Confirmation of Biofilm Formation by Scanning Electron Microscopy (SEM) (Optional)

8h 10m

24 Cut the uncoated polystyrene cell culture microscope slides (Cat. #71888, Electron Microscopy Sciences, Hatfield, Pennsylvania, USA) into approximately 11 mm squares using a scalpel and pliers.

25 Label the polystyrene coupons with a diamond marking pencil (Fisherbrand) and laboratory marker (VWR).

26 Sterilize the coupons with 100% ethanol for 00:20:00 followed by 70% ethanol for 00:20:00 and rinse in sterile MQ water prior to placing them into a sterile polystyrene 24-well plate containing bacterial culture in BB for the formation of biofilms. 40m

27 After biofilm formation, gently rinse the coupons 2 times with [M] 0.1 Mass Percent phosphate buffer (pH 7.4) then fix with 2.5% glutaraldehyde (EM Grade 70% solution, Cat. #16365, Electron Microscopy Sciences) in [M] 0.1 Mass Percent phosphate buffer (7.4).

28 Store the coupons in fixative at 4 °C (minimum 24:00:00) till further processing for SEM.

29 Allow the coupons to gently rotate on a shaker for continuous contact with fresh solution during the fixation and rinsing.

30 After fixation, rinse the coupons 4 times.

30.1 Rinse the coupons with [M] 0.1 Mass Percent phosphate buffer for a minimum of 00:30:00 . (1/4) 30m

30.2 Rinse the coupons with [M] 0.1 Mass Percent phosphate buffer for a minimum of 00:30:00 . (2/4) 30m

30.3 Rinse the coupons with [M] 0.1 Mass Percent phosphate buffer for a minimum of 00:30:00 . (3/4) 30m

30.4 Rinse the coupons with [M] 0.1 Mass Percent phosphate buffer for a minimum of 00:30:00 . (4/4) 30m

30.5 Then, post-fix in 2% osmium tetroxide (Cat. #19134, Electron Microscopy Sciences) in MQ water for 01:00:00 . 1h

31 Rinse the coupons in sterile MQ water for 00:15:00 to remove excess osmium tetroxide then dehydrate in graded series of ethanol (Anhydrous Ethyl Alcohol, Commercial Alcohols, Brampton, Ontario, Canada) as follows. 15m

31.1 Then, dehydrate in 30% ethanol 00:15:00 . 15m

31.2 Dehydrate in 50% ethanol 00:15:00 . 15m

31.3 Dehydrate in 70% ethanol 00:15:00 . 15m

31.4 Dehydrate in 85% ethanol 00:15:00 . 15m

31.5 Dehydrate in 95% ethanol 00:15:00 . 15m

31.6 Dehydrate in 100% ethanol for 01:00:00 . (1/3) 1h

31.7 Dehydrate in 100% ethanol for 01:00:00 .(2/3) 1h

31.8 Dehydrate in 100% ethanol for 01:00:00 .(3/3) 1h

32 Carry out the critical point drying using the Autosamdri – 814 (Rockville, Maryland, USA) critical point dryer.

33 Mount the coupons on 12.7 mm slotted stubs (Cat. #75210, Electron Microscopy Sciences) using silver conductive adhesive (Cat. #12684-15, Electron Microscopy

Sciences) and 12 mm Pelco™ carbon conductive tabs (Cat. #16084-1, Ted Pella Inc., Redding, California, USA).

34 Sputter coat the mounted samples with gold using Emitech K550X Sputter Coater (Ashford, Kent, U.K).

35 Images taken at a Images taken at a View on a SEM (optional: FEI Quanta 600 SEM (Hillsboro, Oregon, USA).

Figure 2. Electronic microscopical confirmation of biofilm formation.

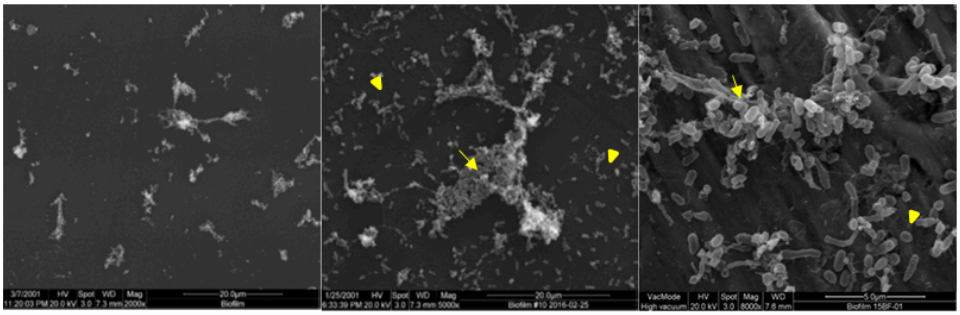

Figure 2. **Electronic microscopical confirmation of biofilm formation**. Images taken at a magnification of ×2000 (image A), x5000 (Image B) and ×8000 (image C) respectively. Arrows indicate cluster of cells and arrow heads indicate adherent cells. **A**: Negative control without bacterial inoculation showing debris from beef broth, scale bars, 20 µm. **B**. Biofilm formation with biofilm OD595 value of 0.491 ± 0.041. scale bars, 20 µm. **C**: Image of biofilms formed on day 9 at 12 ºC in BB on polystyrene coupons. Bacterial cells in clusters and adherent cells in the background can be observed, scale bar, 5µm.

Note

Time Taken
Generally, 10 – 11 days are required for the biofilm assay including 2 days for bacterial culture preparation, and 9 days of incubation for biofilm formation. 2 – 3 days would be required for microscopical and SEM examination.

Expected result

Anticipated results

The biofilm assay uses optical density (OD) measurements to quantify biofilm formation in L. monocytogenes isolates. Based on their respective OD values, these isolates can then be classified into three biofilm-forming phenotypes: strong, moderate, and weak.

Interpretation of results

The biofilm formation capacities are determined arbitrarily in potentially one of the three ways described below or using other methods preferred by users depending on the objectives of different studies.

1. Define the negative, weak, moderate and strong formers by dividing the range between OD_{595} by 3, namely, OD ≤ OD negative control = no biofilm formation, OD_{595} lower 1/3 of the range = weak former, OD_{595} middle 1/3 of the range = moderate former, OD_{595} the upper 1/3 of the range = strong former.
2. If OD ≤ OD of negative control without bacteria (ODC) = no biofilm formation, ODC < OD ≤ (2 × ODC) = weak biofilm formation, (2 × ODC) < OD ≤ (4 × ODC) = moderate biofilm formation, (4 × ODC) < OD = strong biofilm formation (Stepanović et al., 2004; Soosai D, 2017).
3. Compare the biofilm formation capacities of different isolates using individual OD values, which can also be correlated with other measurements including genomics analysis (Soosai et al., 2021).

Protocol references

Reference

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