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Title: Chicken Embryo as an In Vivo Model to Revive Viable but Non-Culturable Pathogens

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Author Questionnaire

1. **Microscopy**: Does your protocol require the use of a dissecting or stereomicroscope for performing a complex dissection, microinjection technique, or something similar? **No**

2. **Software:** Does the part of your protocol being filmed include step-by-step descriptions of software usage? **No**

3. **Filming location:** Will the filming need to take place in multiple locations? **No**

Current Protocol Length
Number of Steps: 25
Number of Shots: 55

Introduction

Videographer: Obtain headshots for all authors available at the filming location.

1.1. **Alessandro Pagliuso:** Our lab is interested in understanding the molecular events regulating bacterial response and adaptation to perturbation in their environment. We use the opportunistic intracellular pathogen *L. monocytogenes* as a bacterial model to understand microbial persistence in different contexts **[1].**

1.1.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera. *Suggested B-roll: 2.4.2*

What are the current experimental challenges?

1.2. **Filipe Carvalho:** The revival of VBNC bacteria is a poorly understood phenomenon. This is particularly evident in Gram-positive species, where dormancy reversal is rarely observed in lab conditions. The shortage of data and effective experimental models presents, therefore, a major challenge to understanding bacterial revival mechanisms **[1]**.

1.2.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera. *Suggested B-roll: 2.5.2*

What significant findings have you established in your field?

1.3. **Filipe Carvalho:** We have shown that *Listeria monocytogenes* sheds its cell wall during the transition to a viable but non-culturable state induced by nutritional and osmotic stress. Importantly, these dormant cell wall-deficient forms remain stable in an aquatic setting and can be revived, constituting potentially dangerous reservoirs of undetectable pathogens **[1]**.

1.3.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera. *Suggested B-roll: 2.7.2*

What research questions will your laboratory focus on in the future?

1.4. **Alessandro Pagliuso:** In light of our findings, we are looking to identify the genetic factors important not only for bacterial transition to a viable but non-culturable state but also for the subsequent revival. Such factors may constitute potential targets for novel strategies aimed at eradicating persistent or undetectable bacterial pathogens **[1]**.

1.4.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera. *Suggested B-roll: 3.1.1* Videographer's NOTE: Use the last one

Videographer: Obtain headshots for all authors available at the filming location.

Protocol

2. Preparation of VBNC (Viable But Non-Culturable) Bacteria

Demonstrator: Filipe Carvalho

2.1. To begin, streak bacterial strains from glycerol stocks stored at minus 80 degrees Celsius onto brain heart infusion agar media using inoculation loops **[1]**. Place the plates in an incubator set to 37 degrees Celsius and incubate overnight to obtain isolated colonies **[2]**.

2.1.1. WIDE: Talent streaking BHI agar plates with an inoculation loop dipped into glycerol stock.

2.1.2. Talent placing the streaked plates into a 37 degrees Celsius incubator.

2.2. Using an inoculation loop, inoculate 2 to 3 colonies from each strain into 2 to 3 tubes containing 5 milliliters of brain heart infusion broth **[1]**. Incubate the cultures overnight at 37 degrees Celsius with shaking at 200 rpm *(R-P-M)* to grow bacteria to the stationary phase **[2]**.

2.2.1. Talent inoculating multiple BHI broth tubes with colonies using a loop.

2.2.2. Talent placing inoculated tubes into a shaking incubator set at 37 degrees Celsius and 200 revolutions per minute.

2.3. The next day, dilute a sample of each overnight culture 1 to 10 in brain heart infusion broth **[1]**. Measure the optical density at 600 nanometers using a spectrophotometer and multiply by 10 to obtain the optical density of the overnight culture **[2-TXT]**.

2.3.1. Talent pipetting 1 part culture into 9 parts BHI broth to make a 1 to 10 dilution. Videographer's NOTE: CU + MED

2.3.2. Talent placing a cuvette into a spectrophotometer and selecting wavelength 600 nanometers. **TXT: Ideal OD_{600}: 2 – 4** Videographer's NOTE: Use take 2

2.4. Pellet 1 milliliter of overnight culture in a 1.5 milliliter microtube at 6,000 *g* for 2 minutes **[1]** and aspirate the supernatant with a vacuum pump **[2]**. Using a pipette, resuspend the cell pellet in 1 milliliter of autoclaved and filtered mineral water **[3-TXT]**.

2.4.1. Talent placing microtubes in a centrifuge and initiating 6,000 times gravity spin.

Videographer's NOTE: Use take 2

2.4.2. Talent removing the supernatant with a vacuum aspirator.

2.4.3. Talent resuspending the pellet with mineral water using a pipette. **TXT: Repeat the centrifugation and resuspension process 3x** Videographer's NOTE: MED + CU

2.5. Prepare bacterial suspensions at a starting concentration of 10^6 colony-forming units per milliliter by adding 30 microliters of washed bacteria into a T25 *(T-twenty-five)* cell culture flask containing 30 milliliters of mineral water **[1]**. Mix the suspensions using a serological pipette **[2]** and store the flask upright at room temperature under dim lighting conditions **[3]**.

2.5.1. Talent pipetting 30 microliters of washed bacteria into a T25 flask containing 30 milliliters of mineral water.

2.5.2. Talent gently mixing the contents of the flask using a serological pipette.

2.5.3. Talent placing the flask upright in a dimly lit area.

2.6. Determine the culturable bacterial population in the suspensions on the first day and then weekly by plating serial dilutions on brain heart infusion agar **[1]**.

2.6.1. Talent taking a sample of suspension from the flask with a micropipette.

2.7. Prepare 10-fold serial dilutions of the bacterial suspension in mineral water ranging from 10^{-1} to 10^{-3} **[1]**. Plate 100 microliters of the undiluted sample and each dilution, in duplicate, on brain heart infusion agar **[2]**. Incubate all plates overnight at 37 degrees Celsius **[3-TXT].**

2.7.1. Talent preparing dilution tubes and pipetting serial dilutions into each. Videographer's NOTE: MED + CU

2.7.2. Talent plating 100 microliters from each dilution onto agar plates in duplicate. Videographer's NOTE: MED + CU

2.7.3. Talent placing all inoculated plates into a 37 degrees Celsius incubator. **TXT: Calculate the concentration of culturable bacteria as CFU/mL**

3. Preparation and Inoculation of Eggs

3.1. Transfer the eggs into the incubator to initiate embryogenesis **[1]**. Position each egg on the tray with the air pocket facing up, so the pointy end is facing downward **[2-TXT]**.

3.1.1. Talent placing eggs onto an incubator tray.

3.1.2. Close-up of talent positioning each egg with the pointed end down. **TXT: Incubation: 37.7 °C; 6 days**

3.2. After 4 days, perform candling to distinguish live embryonated eggs from non-viable ones **[1]**.

3.2.1. Talent taking out trays with eggs for candling inspection.

3.3. Place the egg under a strong light source to illuminate the interior and observe the presence of a blood vessel network **[1]**. In live embryonated eggs, confirm the presence of expanding vessels beneath the air pocket and note any twitching movements **[2]**. Recognize collapsed or absent vessels as indicators of non-viability or absence of embryogenesis **[3]**.

3.3.1. Talent candling an egg using an overhead light and rotating gently.

3.3.2. Show interior of egg with a live embryo and visible vessels.

3.3.3. Show interior of a non-viable egg with collapsed vessels (Videographer's NOTE: Take 2) or of a non-embryonated eggs with absent vessels (Videographer's NOTE: Take 3).

3.4. On the day of inoculation, candle the eggs again to determine the final number of viable and non-viable embryonated eggs, as well as of non-embryonated eggs **[1-TXT]**.

3.4.1. Talent re-candling eggs and sorting them into non-embryonated, viable and non-viable embryonated piles. **TXT: Discard non-viable embryonated eggs**

3.5. Mark the injection spot on each eggshell 2 to 5 millimeters above the air pocket border using a pencil **[1]**. Disinfect the marked area using tissue paper soaked with 70 percent ethanol **[2]**.

3.5.1. Talent marking an egg at the correct height with a pencil.

3.5.2. Talent rubbing the marked shell with ethanol-soaked tissue.

3.6. Now, using an 18-gauge needle, gently create a dent at the injection spot without piercing the inner shell membrane **[1]** and disinfect the spot again with ethanol-soaked tissue paper **[2]**.

3.6.1. Talent gently denting the shell with the needle.

3.6.2. Talent wiping the dented spot with ethanol tissue. Videographer's NOTE: 2nd part

3.7. Next, plug a 25-gauge needle into a 1 milliliter syringe and fill it with the viable but non-culturable bacterial suspension **[1]**.

3.7.1. Talent attaching a 25-gauge needle to the syringe and drawing up VBNC suspension.

3.8. For control conditions, fill syringes with either mineral water or a suspension of *Listeria monocytogenes* grown overnight in brain heart infusion broth at a concentration of 5 x 10^3 colony-forming units per milliliter **[1]**. Insert the needle through the punctured shell at a perpendicular angle until its base touches the shell, positioning the tip in the allantoic cavity or albumen **[2]**.

3.8.1. Talent labeling syringes and drawing up respective control solutions. Videographer's NOTE: Use the last one

3.8.2. Talent inserting the syringe into an egg at a perpendicular angle until the hub touches the shell.

3.9. Now, slowly inject 100 microliters of the inoculum into the egg **[1],** keeping the needle steady during injection to avoid harming the embryo. Next, gently withdraw the needle **[2]** and seal the injection site with a round sticker **[3]**. Use different sticker colors for each condition or group for clarity **[4]**. NOTE: Only sentence numbers are adjusted for the modified shots.

3.9.1. Talent pressing the plunger slowly to inject the inoculum.

3.9.2. Close-up of steady hand and needle position during injection. Talent removing the needle.

3.9.3. Talent ~~removing needle and~~ placing a sticker over the injection site.

3.9.4. Close-up of eggs labeled with color-coded stickers by group.

3.10. Return the inoculated eggs to the incubator and continue incubation for 2 days **[1-TXT]**.

3.10.1. Talent placing labeled eggs back into the incubator. **TXT: Do not expose the eggs**

to temperatures below 37.7 °C for more than 30 min

4. Processing of Embryonated and Non-Embryonated Eggs

Demonstrator: Iana Hemery

4.1. Recover the embryonated eggs from the incubator and candle each one to assess embryo viability **[1]**. Discard eggs with dead embryos and exclude them from the final analysis **[2]**.

4.1.1. Talent bringing eggs to the candling station. Videographer's NOTE: Use take 2

4.1.2. Talent marking and disposing of non-viable eggs into a discard tray.

4.2. Remove the sticker from the egg **[1]** and disinfect the top end of the shell, covering the air pocket, using tissue soaked in 70 percent ethanol **[2]**.

4.2.1. Talent peeling off the egg sticker.

4.2.2. Talent wiping the shell with ethanol-soaked tissue.

4.3. Using clean dissection scissors, cut through the shell from the injection site to reveal the air pocket **[1]**. With clean tweezers, tear open the inner membrane separating the air pocket from the egg interior **[2]**.

4.3.1. Talent cutting the top of the egg open at the marked area. Videographer's NOTE: 4.3.1, 4.3.2, and 4.4.1 are combined. Use Take 2 MED

4.3.2. Talent using tweezers to open the inner membrane gently.

4.4. Now, carefully pour the egg contents into a sterile Petri dish **[1]**. Using tweezers, isolate and transfer the embryo into a new Petri dish **[2]**.

4.4.1. Talent tilting the egg to pour contents into a labeled sterile Petri dish.

4.4.2. Talent isolating the embryo and transferring it, then gently washing it with PBS.

4.5. Transfer the washed embryo into a 15-milliliter centrifuge tube containing 4 milliliters of sterile PBS **[1]**. Homogenize the embryo using a homogenizer set to 10,000 rpm **[2]**. Between samples, clean the dispersion tip sequentially with sterile PBS, 70 percent ethanol, and again sterile PBS **[3]**.

4.5.1. Talent placing embryo into centrifuge tube containing PBS. Videographer's NOTE: Use Take 2

4.5.2. Talent placing embryo under a homogenizer tip.

4.5.3. Talent dipping the homogenizer tip sequentially in PBS, ethanol, and PBS again. Videographer's NOTE: CU + MED

4.6. Plate 500 microliters of the embryo homogenate onto brain heart infusion agar **[1]** and incubate the plate at 37 degrees Celsius overnight or longer, as needed **[2]**. For each inoculation group, count the number of eggs that are positive and negative for *Listeria* growth on agar plates **[3]**.

4.6.1. Talent plating homogenate on labeled BHI agar plate. Videographer's NOTE: MED + CU

4.6.2. Talent placing plates in a 37 degrees Celsius incubator.

4.6.3. Talent examining the plates for colony growth.

4.7. Next, recover the non-embryonated eggs from the incubator **[1]**. Repeat the earlier shell access steps to open the top of the shell and inner membrane **[2]**.

4.7.1. Talent identifying and collecting non-embryonated eggs. Videographer's NOTE: Reuse 4.1.2

4.7.2. Talent opening the egg using scissors and tweezers, exposing the albumen.

4.8. Using a micropipette, collect 500 microliters of albumen from each egg and plate directly onto BHI agar **[1]**. Incubate the plates at 37 degrees Celsius overnight or longer **[2]**. For each inoculation group, count the number of non-embryonated eggs that are positive for *Listeria monocytogenes* growth on agar **[3]**.

4.8.1. Talent pipetting albumen onto labeled BHI agar plates.

4.8.2. Talent transferring plates to the incubator.

4.8.3. Talent examining the plates for colony growth and noting down in a book.

Results

5. Results

5.1. No culturable *Listeria monocytogenes* growth was detected from sterility control eggs injected with mineral water **[1]**, while 100% of eggs injected with culturable *Listeria monocytogenes* tested positive for bacterial growth **[2]**.

5.1.1. LAB MEDIA: Table 1. *Video editor: Highlight the row or column showing zero bacterial growth for the mineral water row and Culturability after egg passage column*.

5.1.2. LAB MEDIA: Table 1. *Video editor: Highlight the row Culturable Lm in column Culturability after egg passage*

5.2. All embryonated eggs injected with viable but non-culturable *Listeria monocytogenes* showed bacterial growth **[1]**, whereas *Listeria* failed to revive in non-embryonated eggs, indicating that revival was not due to lingering culturable bacteria in the inoculum **[2]**.

5.2.1. LAB MEDIA: Table 1. *Video editor: Emphasize the column "embryonated eggs" under the main column "culturability after passage" in "VBNC Lm" row*.

5.2.2. LAB MEDIA: Table 1. *Video editor: Focus on the column "non-embryonated eggs" under the main column "culturability after passage" in "VBNC Lm" row*.

Pronunciation Guide:

1. glycerol

Pronunciation link:
https://www.merriam-webster.com/dictionary/glycerol
en.wikipedia.org+12dictionary.cambridge.org+12howtopronounce.com+12merriam-webster.com+14merriam-webster.com+14openmd.com+14

IPA (American): /ˈglɪs.ə.rɑːl/
Phonetic spelling: glis-uh-rawl

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2. spectrophotometer

No confirmed link found. This is a common scientific instrument name without a unique entry—but pronunciation is standard:
IPA (American): /ˌspɛk·troʊ·fəˈtɑː·mɪ·tər/
Phonetic spelling: spek-troh-foh-TAH-muh-ter

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3. nanometers

No confirmed link found. Standard term derived from “nano-” + “meter”:
IPA: /næˈnɒm·ɪ·tərz/ (American commonly: /næˈnoʊ·mɪ·tərz/)
Phonetic spelling: nan-OH-mee-terz

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4. centrifuge

No confirmed link found. Common lab term:
IPA: /ˈsɛn·trəˌfjuʒ/
Phonetic spelling: SEN-truh-fyooj

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5. aspirate

No confirmed link found. In lab context meaning remove fluid:
IPA: /ˈæs·pəˌreɪt/
Phonetic spelling: AS-puh-rayt

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6. serological

No confirmed link found. Derived from “serology”:
IPA: /ˌsɛr·əˈlɑː·dʒɪ·kəl/
Phonetic spelling: ser-uh-LOH-ji-kul

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7. embryonated

No confirmed link found. From “embryonate”:
IPA: /əmˈbraɪ·əˌneɪ·tɪd/
Phonetic spelling: em-bry-oh-NAY-tid

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8. allantoic

Pronunciation link:
https://www.merriam-webster.com/medical/allantoic dictionary.cambridge.org+14merriam-webster.com+14merriam-webster.com+14merriam-webster.com+3merriam-webster.com+3en.wikipedia.org+3

IPA: /ˌæl·ənˈtoʊ·ɪk/
Phonetic spelling: al-uhn-TOH-ik

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9. albumen

No confirmed link found. Common term for egg white:
IPA: /ælˈbjuː·mən/
Phonetic spelling: al-BYOO-muhn

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10. homogenizer

No confirmed link found. Device for blending:
IPA: /həˈmɑː·dʒəˌnaɪ·zər/
Phonetic spelling: huh-MAH-juh-ny-zer

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11. Listeria monocytogenes

No confirmed link found. Scientific name:
IPA: /lɪˈstɪr·i·ə/ for *Listeria*; /ˌmɑːˌnəˌsaɪ·təˈdʒɛniz/ for *monocytogenes*
Phonetic spelling: lis-TEER-ee-uh mon-oh-sy-TOH-juh-neez
