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protocols for Isolation, Characterization, and Therapeutic Application of Bacteriophages for MRSA Mastitis

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We use this protocol and it's working

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Abstract

This protocol describes the isolation, characterization, and application of a novel bacteriophage cocktail (SW21-SW25) for treating MRSA bovine mastitis. The cocktail, containing two newly isolated phages (vB_SauR_SW21 and vB_SauR_SW25), effectively reduced bacterial burden in a mouse model, displaying efficacy comparable to antibiotics. **Additionally, the treatment decreased pro-inflammatory cytokines (IL-1β and TNF-α) compared to the control group.**

Guidelines

- Ahmadi-Noorbakhsh, S., Mirabzadeh Ardakani, E., Sadighi, J. *et al.* Guideline for the Care and Use of Laboratory Animals in Iran. *Lab Anim* **50**, 303–305 (2021). https://doi.org/10.1038/s41684-021-00871-3. https://doi.org/10.1038/s41684-021-00871-3

Troubleshooting

Preparing Brain Heart Infusion (BHI) Agar

1 **Preparation:**

- Determine the required amount of dehydrated BHI powder based on the desired volume and manufacturer's instructions.
- Mix the powder with distilled water to create a homogeneous solution.

Sterilization:

- Boil the solution to ensure complete dissolution of the powder.
- Sterilize the solution using an autoclave to eliminate any contaminants.

Cooling and Pouring:

- Allow the autoclaved media to cool to a suitable temperature (approximately 50 °C).
- Distribute the cooled media into sterile Petri dishes.

Incubation: Incubate the Petri dishes at 37 °C for Overnight to observe for any signs of contamination.
Storage: If no contamination is evident, refrigerate the plates for future use.
Purpose: Prepared BHI agar is used for:

- **Isolation:** Isolating specific bacterial strains from samples.
- **Subculture:** Transferring bacteria to fresh media for continued growth and study.

Preparing Mueller-Hinton Agar (MHA)

2 **Preparation:** 1d

- **Dissolve:** Prepare the media by dissolving a specific amount of dehydrated powder in distilled water following the manufacturer's instructions.
- **Adjust pH:** Ensure the pH of the prepared medium is within the optimal range of pH 7.2 to pH 7.4

Sterilization:

- **Heat and Dissolve:** Boil the medium to ensure complete dissolution of the powder and homogeneity.
- **Autoclave:** Sterilize the medium using an autoclave to eliminate contaminants.

Cooling and Pouring:

- **Cool:** Allow the autoclaved media to cool to a suitable temperature (approximately 50 °C).
- **Pour:** Distribute the cooled media into sterile Petri dishes, maintaining a depth of 4 mm (as per CLSI guidelines) for accurate antibiotic susceptibility testing (AST) using the disk diffusion method.

Incubation: Incubate the Petri dishes at 37 °C for 24:00:00 to observe for any signs of contamination.
Storage: If no contamination is evident, refrigerate the plates for future use in antibiotic susceptibility testing.
Purpose: Prepared Mueller-Hinton agar is specifically designed for antibiotic susceptibility testing and determining the effectiveness of various antibiotics against different bacterial strains.

Preparing 1x LB Broth

3 **Preparation:**

- **Dissolve:** Prepare the media by dissolving a specific amount of dehydrated powder in distilled water following the manufacturer's instructions.

Sterilization:

- **Heat and Dissolve:** Boil the medium to ensure complete dissolution of the powder and homogeneity.
- **Autoclave:** Sterilize the medium using an autoclave to eliminate contaminants.

Cooling and Storage:

- **Cool:** Allow the autoclaved media to cool to a suitable temperature.
- **Store:** Refrigerate the cooled media for future use.

Purpose: 1x LB broth is a versatile medium used for:

- **Bacterial Cultures:** Growing bacterial cultures overnight.
- **Phage Cultures:** Performing sequential cultures of bacteriophages.
- **Phage Stock Preparation:** Preparing serial dilutions of phage stock.

Preparing 2x LB Broth

4 **Preparation:**

- **Weigh:** Measure the amount of LB broth powder based on your desired final volume.
- **Dissolve:** Prepare a concentrated 2x LB broth by adding half the intended volume of
distilled water.

Sterilization:

- **Heat and Dissolve:** Boil the medium to ensure complete dissolution of the powder and homogeneity.
- **Autoclave:** Sterilize the medium using an autoclave to eliminate contaminants.

Cooling and Storage:

- **Cool:** Allow the autoclaved media to cool to a suitable temperature.
- **Store:** Refrigerate the cooled media for future use.

Purpose: Concentrated 2x LB broth is used for:

- **Enrichment:** Increase the population of specific bacteriophages in wastewater samples.
- **Detection:** Facilitate the easier detection and study of bacteriophages.

Preparing Bottom Agar (LB broth + 1.5 % (w/v) agar)

5 **Preparation:**

- **Dissolve:** Prepare the base by dissolving LB broth powder in distilled water.
- **Add Agar:** Incorporate agar powder, constituting 1.5% of the final medium volume, into the liquid base.

Heating and Sterilization:

- **Heat:** Boil the mixture to dissolve the powder and agar.
- **Autoclave:** Sterilize to eliminate contaminants.

Dispensing: Pour the cooled medium into sterile Petri dishes, ensuring a thin layer.

Purpose: Prepared LB agar plates provide a nutrient-rich environment for:

- **Double Layer Agar (DLA) test:** Drop lawn assay, used to enumerate bacterial or viral populations.
- **Spot Assay:** Characterizing bacterial or viral populations.

Preparing Top Agar (LB broth + 0.7 % (w/v) agar)

6 **Preparation:**

- **Dissolve:** Prepare the base by dissolving LB broth powder in distilled water.
- **Add Agar:** Incorporate agar powder to create a solid medium.

Heating and Sterilization:

- **Heat:** Boil the mixture to dissolve all components.
- **Autoclave:** Sterilize the mixture to eliminate contaminants.

Calcium Chloride Addition: Incorporate a specific amount of 1 M calcium chloride solution to achieve a final concentration of 2 mM.

Storage: Refrigerate the prepared medium until needed.

Usage: Before each experiment, melt the medium and maintain it at 50 °C to prevent solidification.

Purpose: Prepared LB agar plates with calcium chloride are used for:

- **DLA:** Drop lawn assay, used to enumerate bacterial or viral populations.
- **Spot Assays:** Characterizing bacterial or viral populations.

Preparing TBE Buffer

7 **Preparation of 5x TBE stock solution:**

1. Measure the required amounts of EDTA (4.63 g), Tris base (54 g), and boric acid (27.5 g).

2. Dissolve the weighed powders in distilled water (800 mL).
3. Adjust the pH of the solution to the desired level (pH 8.3).
4. Bring the final volume up to 1 liter.

Dilution for Use: Dilute the 5x TBE stock solution 10-fold with distilled water to prepare a 0.5x TBE working solution.

Applications of 0.5x TBE:

- Preparing agarose gels for gel electrophoresis.
- Buffer for the electrophoresis tank.

Preparation of 1% Agarose Gel for Electrophoresis

8 **Materials:**

- Agarose powder
- 0.5x TBE buffer
- Electrophoresis tank
- Comb
- Safe stain (optional)
- Glass mold
- Weighing scale

Procedure:

1. **Calculate volume:** Determine the required volume of agarose gel based on the mold dimensions and desired gel thickness.
2. **Weigh powder:** Accurately weigh the calculated amount of agarose powder using a precise scale.
3. **Mix with buffer:** Add the weighed agarose powder to the calculated volume of 0.5x TBE buffer.
4. **Dissolve:** Heat the mixture to dissolve the agarose powder completely.
5. **Cool:** Allow the gel to cool to a suitable temperature.
6. **Add stain (Optional):** Incorporate a safe stain into the cooled gel for visualization.
7. **Pour gel:** Pour the cooled gel into the glass mold.
8. **Insert comb:** Immediately insert the comb into the gel to create sample wells.
9. **Remove comb:** Once the gel has been set, carefully remove the comb.
10. **Transfer to tank:** Place the gel in the electrophoresis tank filled with 0.5x TBE buffer.

Note: Ensure the buffer in the tank matches the buffer used to prepare the gel.

Gram Staining

9 **Materials:**

- Glass slide
- Normal saline (0.9% (w/v) NaCl)
- Pure bacterial colony

- Crystal violet
- Lugol's iodine
- Alcohol
- Safranin dye
- Light microscope
- 100x objective lens
- Immersion oil

Procedure:

1. **Prepare Smear:** Place a drop of physiological serum on a glass slide and add a small amount of the pure bacterial colony. Spread the mixture to create a thin film.
2. **Air Dry:** Allow the smear to air dry completely.
3. **Heat Fix:** Pass the slide over a flame two or three times to fix the bacteria.
4. **Apply Crystal Violet:** Cover the smear with crystal violet and incubate for 1 minute.
5. **Wash:** Rinse the slide thoroughly with water.
6. **Apply Lugol's Iodine:** Add Lugol's iodine and incubate for 30 seconds.
7. **Wash:** Rinse the slide thoroughly with water.
8. **Decolorize:** Apply alcohol for 15 seconds to decolorize.
9. **Wash:** Rinse the slide thoroughly with water.
10. **Apply Safranin:** Add safranin dye and incubate for 45 seconds.
11. **Wash:** Rinse the slide thoroughly with water.
12. **Dry:** Allow the slide to air dry completely.
13. **Microscopic Examination:** Examine the slide under a light microscope using a 100x objective lens and immersion oil. Observe the morphology of the bacteria.

Note: Gram-positive bacteria, such as *Staphylococcus aureus*, will appear purple under the microscope, while Gram-negative bacteria will appear pink.

Catalase Test

10 **Materials:**

- Glass slide
- 3% hydrogen peroxide solution
- Swab or non-metallic ounce
- Bacterial isolates

Procedure:

1. **Place Hydrogen Peroxide:** Place a drop of 3% hydrogen peroxide solution on a glass slide.
2. **Add Bacteria:** Using a swab or non-metallic ounce, remove a small amount of the bacterial colony an mix it with the hydrogen peroxide solution.
3. **Observe Bubbles:** Observe the mixture for the formation of oxygen bubbles.

Interpretation:

- **Positive Test:** The appearance of oxygen bubbles indicates a positive catalase test.
- **Negative Test:** The absence of bubbles indicates a negative catalase test.

Note: *Staphylococcus aureus* is catalase-positive.

Coagulase Test

11 **Materials:**

- Rabbit citrated plasma
- Sterile normal saline (0.9% (w/v) NaCl)
- Sterile test tubes
- Loop
- Standard bacterial colony (coagulase-positive control)
- Water bath (37°C)

Procedure:

1. **Dilute Plasma:** Dilute rabbit-citrated plasma 1:5 with sterile normal saline.
2. **Prepare Test Tubes:** Add 0.5 mL of diluted plasma to each sterile test tube.
3. **Add Bacteria:** Add a small amount of the tested bacterial colony to each tube and mix using a loop.

Set Up Controls:

- **Negative Control:** Add sterile normal saline to a tube containing the standard bacterial colony.
- **Positive Control:** Add the standard coagulase-positive bacterial colony to diluted plasma.

4. **Incubate:** Incubate all tubes at 37°C.
5. **Monitor Coagulation:** Check tubes for coagulation every 30 minutes for up to four hours.
6. **Extended Incubation:** If no coagulation is observed after four hours, incubate tubes at room temperature for 24 hours.
7. **Interpret Results:** A positive coagulase test is indicated by clot or gel formation in the tube.

Note: *Staphylococcus aureus* is typically coagulase-positive

DNase Test

12 **Materials:**

- DNase agar culture medium

- HCl (1 N)
- Bacterial isolate

Procedure:

1. **Prepare Culture:** Perform a linear culture of the desired bacteria on DNase agar culture medium.
2. **Incubate:** Incubate the culture at 37°C for 18-24 hours.
3. **Add HCl:** Apply a few drops of 1 N HCl to the surface of the culture medium.
4. **Observe Halo:** Check the area around the bacterial culture line for a clear halo.

Interpretation:

- **Positive Test:** A clear halo around the culture line indicates DNA hydrolysis and DNase enzyme.
- **Negative Test:** The absence of a halo suggests no DNase production.

Note: *Staphylococcus aureus* is typically DNase-positive.

Mannitol Fermentation Test

13 **Materials:**

- Mannitol salt agar culture medium
- Bacterial isolate

Procedure:

1. Perform a linear culture of the desired bacteria on mannitol salt agar.
2. Incubate the culture at 37°C for 18-24 hours.
3. Examine the culture medium for bacterial growth and any color change.

Interpretation:

- Growth on mannitol salt agar indicates salt tolerance.
- A color change from red to yellow indicates mannitol fermentation and acid production.

Note: *Staphylococcus aureus* is salt-tolerant and capable of fermenting mannitol, resulting in growth and a yellow color change on mannitol salt agar.

Bacterial Stock Preparation for Long-Term Storage

14 **Materials:**

- LB broth containing 20% glycerol
- 18-hour bacterial culture
- Loop
- Vortex mixer

- -70°C freezer

Procedure:

1. **Prepare Stock:** Inoculate a loopful of 18-hour bacterial culture into LB broth containing 20%
   glycerol.
2. **Incubate:** Incubate the mixture at 37°C for two hours.
3. **Vortex:** Vortex the sample for a few seconds to ensure homogeneity.
4. **Store:** Transfer the vial to a -70°C freezer for long-term storage.

Methicillin Resistance Testing (Cefoxitin Disk Diffusion)

15 **Materials:**

- Pure bacterial culture
- Sterile normalsaline (0.9% (w/v) NaCl)
- 0.5 McFarland standard (OD600 = 0.08- 0.13)
- Sterile cotton swab
- Mueller-Hinton agar
- Cefoxitin disk (30 µg)
- Incubator (33-35°C)
- Ruler
- MRSA ATCC 43300
- Staphylococcus aureus ATCC 25923

Procedure:

1. Dilute pure bacterial culture in sterile normal saline to achieve a turbidity equal to 0.5 McFarland standard.
2. Using a sterile cotton swab, inoculate Mueller-Hinton agar in a lawn pattern with the bacterial suspension.
3. Place a cefoxitin disk (30 µg) in the center of the inoculated agar.
4. Incubate the plate at 33-35°C for 16-18 hours.
5. Measure the diameter of the zone of inhibition (lack of growth) around the cefoxitin disk.

Interpret Results:

- **Resistant:** If the diameter is ≤ 21 mm, the isolate is resistant to methicillin.
- **Sensitive:** If the diameter is ≥ 22 mm, the isolate is sensitive to methicillin.

Control Strains: Use MRSA ATCC 43300 and Staphylococcus aureus ATCC 25923 as positive and negative controls, respectively.

Note: Follow CLSI guidelines for specific breakpoint values and interpretation criteria.

Bacterial Genome Extraction (Boiling Method)

16 **Materials:**

- Fresh bacterial culture
- Sterile distilled water
- Shaker
- Water bath (100°C)
- Centrifuge (12000 rpm)
- New vial
- Refrigerator (4°C)

Procedure:

1. Obtain a fresh culture of the desired bacteria on BHI agar.
2. Inoculate pure bacterial colonies into a vial containing 400 µL of sterile distilled water.
3. Shake the suspension to ensure uniformity.
4. Place the samples in a water bath at 100°C for 12 minutes.
5. Centrifuge the samples at 12000 rpm for 10 minutes.
6. Transfer the liquid phase (supernatant) to a new vial.
7. Store the extracted DNA in the refrigerator at 4°C.

mecA Gene-Specific PCR for Methicillin Resistance Confirmation

17 **Materials:**

- Genomic DNA
- Taq 2× master mix
- Forward primer (F-TGGCCAATTCCACATTGTTTCG)
- Reverse primer (R-TCCAGGAATGCAGAAAGACCA)
- Sterile distilled water
- PCR tubes
- Thermocycler
- 1% agarose gel
- Electrophoresis chamber
- UV light

Procedure:

1. **Prepare Reaction Mixture:** In each PCR tube, combine the following:

- 7.5 µL Taq 2× master mix
- 0.5 µL forward primer
- 0.5 µL reverse primer
- 2 µL template DNA
- 4.5 µL sterile distilled water

2. **Set Up PCR Conditions:** Program your thermocycler with the following conditions:
   - Initial denaturation: 95°C for 5 minutes
   - 30 cycles of:
   - Denaturation: 95°C for 40 seconds
   - Annealing: 60°C for 30 seconds
   - Extension: 72°C for 30 seconds
   - Final extension: 72°C for 5 minutes
3. **Run PCR:** Perform the PCR amplification using the programmed conditions.
4. **Gel Electrophoresis:** Load the PCR products onto a 1% agarose gel and run electrophoresis at 120 V for 45 minutes.
5. **Visualize:** Visualize the PCR products under UV light.

Interpretation:

- **Positive Result:** A PCR product of the expected size indicates the presence of the *mecA* gene and confirms methicillin resistance.
- **Negative Result:** The absence of a PCR product suggests the absence of the *mecA* gene and susceptibility to methicillin.

Sample Preparation for Phage Isolation

18 **Materials:**

- Solid sample (e.g., manure, feces)
- 1x LB broth
- Falcon tube
- Shaking incubator (37°C, 150 rpm)
- Filter paper
- Centrifuge
- 0.22 µm pore-size sterile filter
- Storage container (4°C)

Procedure:

Solid Samples:

1. Combine 0.5 g of solid sample with 4.5 mL of 1x LB broth in a falcon tube.
2. Invert the tube to ensure complete mixing.
3. Incubate the mixture in a shaking incubator at 37°C and 150 rpm for 2 hours.
4. Filter the incubated mixture through filter paper to remove solids.

All Samples (Wastewater or Processed Solid Sample Suspensions):

1. Centrifuge the sample at 8,000 rpm for 15 minutes at 20°C.
2. Pass the supernatant through a 0.22 µm pore-size sterile filter.
3. Store the sterile filtrate at 4°C.

Bacteriophage Enrichment

19 **Materials:**

- *Staphylococcus aureus* ATCC 43300 (MRSA host strain)
- BHI agar
- 1x LB broth
- 2x LB broth containing 2 mM $CaCl_2$
- Filtered wastewater sample
- Shaking incubator (37°C, 150 rpm)
- Centrifuge (8,000 rpm, 4°C)
- 0.22 µm pore-size filter

Procedure:

1. Streak the MRSA strain on BHI agar and incubate overnight to obtain a pure culture.
2. Inoculate a single colony from the fresh culture into 1x LB broth and incubate overnight at 37°C with shaking.
3. Combine 0.1 mL of the overnight bacterial culture (OD600 = 0.5) with 10 mL of 2x LB broth containing $CaCl_2$ and 10 mL of filtered wastewater.
4. Incubate the mixture in a shaking incubator at 37°C and 150 rpm for 18-20 hours.
5. Centrifuge the suspension at 8,000 rpm and 4°C for 15 minutes.
6. Filter the supernatant through a 0.22 µm pore-size filter.
7. Store the lysate (filtrate containing enriched phages) at 4°C.

Spot Assay for Phage Detection

20 **Materials:**

- *S. aureus* ATCC 43300 (OD600 = 0.05)
- Molten top agar (LB broth with 0.7% (w/v) agar and 2 mM $CaCl_2$)
- Solidified bottom agar (LB broth with 1.5% (w/v) agar)
- Lysate
- Incubator(37°C)

Procedure:

1. Prepare an overnight culture of *S. aureus* ATCC 43300 to an OD600 of 0.05.
2. Mix 0.1 mL of the bacterial suspension with molten top agar.
3. Pour the mixture onto solidified bottom agar.
4. Spot 10 µL of lysate onto the center of the overlaid lawn.
5. Incubate the plate at 37°C for 24 hours.
6. Examine the plate for clear zones (plaques), indicating lytic phage activity against MRSA.

Bacteriophage Isolation and Purification

21 **Materials:**

- Lysate containing bacteriophages
- *S. aureus* ATCC 43300 (OD600 = 0.5)
- Molten top agar
- Solidified bottom agar plates
- Sterile pipette tip
- Incubator (37°C)

Procedure:

1. Prepare a ten-fold serial dilution of the lysate (10^{-1} to 10^{-9}).
2. Mix 0.1 mL of each dilution with 0.1 mL of overnight *S. aureus* ATCC 43300 culture and molten top agar.
3. Pour the mixture onto solidified bottom agar plates.
4. Incubate the plates at 37°C for 18-24 hours.
5. Identify distinct plaques on the plates.
6. Gently pick a single phage plaque with a sterile pipette tip and streak it onto a new plate.
7. Repeat the streaking and overlay process up to three times to ensure pure phage clones.

Note: Ensure gentle handling to avoid damaging the phage particles during the streaking process.

Phage Propagation

22 **Materials:**

- Pure phage plaque
- Sterile pipette tip
- 1x LB broth
- Sterile microtubes
- Falcon tubes
- *S. aureus* ATCC 43300 (OD600 = 0.5)
- $CaCl_2$ (2 mM)
- Centrifuge (8000 rpm)
- 0.2 µm pore-size filter
- Molten top agar (LB broth with 0.7% (w/v) agar and 2 mM $CaCl_2$)
- Solidified bottom agar (LB broth with 1.5% (w/v) agar)

Procedure:

1. Remove a selected phage plaque from the medium using a sterile pipette tip.

2. Transfer the plaque to a microtube containing 1 mL of 1x LB broth and incubate overnight at room temperature.
3. Add the contents of the microtube to a Falcon tube containing 9 mL of *S. aureus* ATCC 43300 (OD600 = 0.5) and $CaCl_2$ (2 mM). Incubate for 24 hours.
4. Centrifuge at 8000 rpm for 15 minutes. Filter the supernatant using a 0.22 µm pore-size filter to sterilize the lysate.

Phage Titer Determination (DLA Method)

23 **Materials:**

- Molten top agar (LB broth with 0.7% (w/v) agar and 2 mM $CaCl_2$)
- Solidified bottom agar (LB broth with 1.5% (w/v) agar)
- Lysate containing bacteriophages
- Dilution tubes

Procedure:

1. Perform the double-layer agar assay as described previously.
2. After incubation, count the number of plaques on each dilution plate.
- Calculate PFU/ml: Multiply the plaque count by 10.
- Divide the result by the dilution factor.

3. Report Titer: Report the titer of bacteriophages present in the stock as the PFU/ml value from the dilution range of 30-300 plaques.

Phage Storage

24 **For Short-Term Storage:**
Store the phage suspension in 1x LB broth at 4°C.

For Long-Term Storage:

1. **Add Glycerol:** Mix the phage suspension with 20% glycerol.
2. **Store at -70°C:** Transfer the mixture to a freezer and store at -70°C.

Host Range Determination of Bacteriophages

25 **Materials:**

- Isolated bacteriophages
- MRSA and MSSA isolates
- 1x LB broth
- Molten top agar (LB broth with 0.7% (w/v) agar and 2 mM $CaCl_2$)
- Solidified bottom agar (LB broth with 1.5% (w/v) agar)
- Incubator (37°C)

Procedure:

1. Prepare overnight cultures of MRSA and MSSA isolates in 1x LB broth.
2. Prepare double-layer agar plates as described previously.
3. Spot 10 µl of the lysate-containing bacteriophages onto the center of the overlaid lawn.
4. Incubate at 37°C for 18-24 hours.
5. Examine the plates for the presence of plaques.

Interpretation:

- **Sensitivity:** The presence of plaques indicates bacterial sensitivity to the bacteriophages.
- **Resistance:** The absence of plaques indicates bacterial resistance to the bacteriophages

Efficiency of Plating (EOP) Determination

26 **Materials:**

- Phage-sensitive clinical isolates (identified by spot test)
- Host strain (*S. aureus* ATCC 43300)
- 1x LB broth
- Molten top agar (LB broth with 0.7% (w/v) agar and 2 mM $CaCl_2$)
- Solidified bottom agar (LB broth with 1.5% (w/v) agar)

Procedure:

1. Prepare overnight cultures of the phage-sensitive clinical isolates and the host strain in 1x LB broth.
2. Using the DLA method determine phage titer (PFU/mL) for each clinical isolate and the host strain.
3. **Calculate EOP:** Divide the phage titer in the clinical isolate by the phage titer in the host strain.
4. **Classify Bacteriophage:**

- **Highly virulent:** 0.1 < EOP < 1.00
- **Moderately virulent:** 0.001 < EOP < 0.099
- **Avirulent but active:** EOP < 0.001
- **Avirulent:** No plaques detected.

Note: The EOP value indicates the phage's ability to replicate and infect the host bacteria.

Optimal Multiplicity of Infection (MOI) Determination

27 **Materials:**

- *S. aureus* ATCC 43300 (10^6 CFU/mL)

- 1x LB broth containing 2 mM $CaCl_2$
- Centrifuge (5,000 x g)
- Molten top agar (LB broth with 0.7% (w/v) agar and 2 mM $CaCl_2$)
- Solidified bottom agar (LB broth with 1.5% (w/v) agar)

Procedure:

1. Prepare a 10-fold serial dilution of bacteriophages in 1x LB broth containing 2 mM $CaCl_2$.
2. Combine equal volumes of each phage dilution with the host strain culture (10^6 CFU/mL).
3. Incubate the mixtures at 37°C with shaking for 15 minutes.
4. Centrifuge the suspensions at 5,000 × g for 10 minutes.
5. Resuspend the pellet in 1 mL of 1x LB broth with 2 mM CaCl2.
6. Incubate the resuspended pellets at 37°C with shaking for 4 hours.
7. Determine the phage titer in each sample using the DLA method.
8. Divide the phage titer by the number of host bacteria (10^6 CFU/mL) to calculate the MOI.
9. The optimal MOI is the ratio that results in the highest phage titer.

Transmission Electron Microscopy (TEM)

28 **Materials:**

- High-titer bacteriophage suspension (10^{10} PFU/mL)
- Centrifuge (25,000 × g)
- Ammonium acetate buffer (0.1 M, pH 7.2)
- Carbon-coated copper grid
- 2% uranyl acetate stain
- Filter paper
- Transmission electron microscope

Procedure:

1. Centrifuge the phage suspension at 25,000 × g for 60 minutes.
2. Discard the supernatant and resuspend the pellet in 1 mL of ammonium acetate buffer.
3. Repeat the centrifugation and resuspension steps twice more.
4. Apply 10 µL of the concentrated phage suspension to a carbon-coated copper grid.
5. Incubate the grid with 2% uranyl acetate stain for one minute.
6. Remove excess stain with filter paper and rinse with distilled water.
7. Allow the grid to air dry.
8. Examine the prepared TEM sample using a transmission electron microscope at 100 kV.

Bacteriophage Stability Evaluation

29 **Materials:**

- Bacteriophage suspension (10^9 PFU/mL)
- 1x LB broth
- Water bath (various temperatures)
- NaOH and HCl (for pH adjustment)
- Molten top agar (LB broth with 0.7% (w/v) agar and 2 mM $CaCl_2$)
- Solidified bottom agar (LB broth with 1.5% (w/v) agar)

Procedure:

Temperature Stability:

1. Incubate bacteriophage suspension at various temperatures (37°C, 50°C, 60°C, and 70°C).
2. Collect samples at 5, 15, 30, 60 minutes, and 24 hours.
3. Determine phage titer using the DLA method.

pH Stability:

1. Prepare bacteriophage suspension in 1x LB broth with varying pH (3 to 11, adjusted with NaOH or HCl).
2. Incubate the samples at 37°C for one hour.
3. Determine phage titer using the DLA method.

Note: All experiments should be conducted in duplicate for reproducibility.

Phage Adsorption Rate Determination

30 **Materials:**

- *S. aureus* ATCC 43300 (logarithmic growth phase, OD600 = 0.1-0.2)
- 1x LB broth
- Phag suspension (10^7 PFU/mL)
- Shaker (60 rpm)
- Centrifuge (16,000 × g)
- Molten top agar (LB broth with 0.7% (w/v) agar and 2 mM $CaCl_2$)
- Solidified bottom agar (LB broth with 1.5% (w/v) agar)

Procedure:

1. Culture host bacteria to logarithmic growth phase and dilute to OD600 = 0.1-0.2.
2. Determine bacterial concentration (CFU/mL) using the colony count method.
3. Combine 9 mL of bacterial suspension with 1 mL of phage suspension (MOI = 0.01).
4. Incubate the mixture at 37°C with shaking for 60 rpm.

5. Withdraw 50 µL aliquots every minute and dilute them in 950 µL of 1x LB broth. Vortex and keep on ice.
6. Centrifuge the diluted aliquots at 16,000 × g for 10 minutes. Titrate the supernatant using the DLA method to quantify unbound phages.
7. **Calculate Adsorption Rate Constant (k):** Use the following formula, where B is the initial bacterial density, P0 is the initial phage concentration, P is the final phage concentration, and t is the time taken for the phage titer to drop.

$$K = 2.3/Bt \log P0/P$$

Note: Use 1x LB broth as a negative control.

One-Step Growth Curve Assay

31 **Materials:**

- *S. aureus* ATCC 43300 (supplemented with 2 mM $CaCl_2$)
- Bacteriophage suspension (10^6 PFU/mL)
- Centrifuge (7,500 × g)
- 1x LB broth containing 2 mM $CaCl_2$
- Molten top agar (LB broth with 0.7% (w/v) agar and 2 mM $CaCl_2$)
- Solidified bottom agar (LB broth with 1.5% (w/v) agar)

Procedure:

1. Combine 900 µL of S. aureus ATCC 43300 (supplemented with 2 mM CaCl2) with 100 µL of bacteriophage suspension.
2. Incubate the mixture at 37°C for 10 minutes.
3. Centrifuge the mixture at 7,500 × g for 5 minutes.
4. Resuspend the pellet in 1 mL of fresh 1x LB broth with 2 mM $CaCl_2$.
5. Incubate the resuspended pellet at 37°C with shaking for 90 minutes.
6. Collect 100 µL aliquots every 5 minutes.
7. Determine the phage titer in each aliquot using the DLA method.

Phage Genome Extraction using Phage DNA extraction kit (Norgen Biotek, Canada)

32 **Materials:**

- Phage suspension (10^{10} PFU/mL)
- DNase I enzyme
- Lysis buffer
- Proteinase K enzyme
- Isopropanol
- Nuclease Free microtubes
- Washing buffer
- Elution buffer

- Centrifuge (14,000 × g and 6,000 × g)
- Vortex
- Water bath

Procedure:

1. Centrifuge the phage suspension at 14,000 × g for 45 minutes and resuspend the pellet in 1 mL of 1x LB broth.
2. Add 20 µL of DNase I enzyme to the concentrated sample and incubate at 37°C for 30 minutes, followed by 10 minutes at 65°C.
3. Add 500 µL of lysis buffer (pre-incubated at 40°C) and vortex.
4. Add 4 µL of proteinase K enzyme and incubate at 50°C for 30 minutes, followed by 15 minutes at 65°C.
5. Add 320 µL of isopropanol and vortex.
6. Pass the mixture through the column in three steps, centrifuging at 6,000 × g for one minute.
7. Wash the column three times with washing buffer, centrifuging at 6,000 × g for one minute each time.
8. Centrifuge the column at 14,000 ×g for two minutes to dry the filter. Transfer the column to a new microtube and add 150 µL of elution buffer, centrifuging at 6,000 × g for one minute.
9. Store the eluted phage genome at -20°C.

Note: Ensure the lysis buffer is pre-incubated at 40°C as instructed. Mix the sample well during the proteinase K treatment step.

Evaluation of Extracted DNA Quality

33 **Materials:**

- Extracted genomic DNA (5 µL)
- 1% agarose gel
- Nanodrop device

Procedure:

Qualitative Evaluation:

1. **Gel Electrophoresis:** Run 3 µL of extracted DNA on a 1% agarose gel to visualize DNA integrity and size.

Quantitative Evaluation:

1. **Measure Absorbance:** Use a Nanodrop device to measure the absorbance of the DNA samples at 260 nm and 280 nm.
2. **Calculate DNA Concentration:** Determine the DNA concentration in ng/µL based on the absorbance at

260 nm.

3. **Evaluate Purity:**

- **High Purity:** 260/280 A ratio between 1.7 and 2.0 indicates high DNA purity.
- **Protein Contamination:** 260/280 A ratio less than 1.7 suggests protein contamination.
- **RNA Contamination:** 260/280 A ratio greater than 2.0 indicates RNA contamination.

Note: A high-quality DNA sample should have a clear, distinct band on the agarose gel and a 260/280 A ratio within the acceptable range.

Whole Genome Sequencing Analysis of Isolated Bacteriophages

34 **Materials:**

- Isolated bacteriophages
- Phage DNA extraction kit (Norgen Biotek, Canada)
- Illumina sequencing platform
- Galaxy server (https://usegalaxy.eu)
- Proksee server (**https://proksee.ca/**)
- CGE server (https://www.genomicepidemiology.org/)

Software:

- FastQC (Galaxy Version 0.74+galaxy0)
- Shovill (Spades) tool (Galaxy Version 1.1.0+galaxy2)
- RAST server (https://rast.nmpdr.org/rast.cgi)
- BLASTP (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE=Proteins)
- tRNAscan-SE (Galaxy Version 2.0.5)
- Phage AI tool (**https://www.phage.ai/**)
- CARD (https://card.mcmaster.ca/)
- ResFinder (http://genepi.food.dtu.dk/resfinder)
- VFDB (http://www.mgc.ac.cn/VFs/)
- Easyfig software (https://mjsull.github.io/Easyfig/)
- VIRIDIC tool (https://rhea.icbm.uni-oldenburg.de/viridic/)
- VICTOR (Virus Classification and Tree Building Online Resource) (https://ggdc.dsmz.de/victor.php)

Procedure:

1. **DNA Extraction:** Extract genomic DNA from the isolated bacteriophages using the Phage DNA extraction kit following the manufacturer's instructions.
2. **Sequencing:** Perform whole genome sequencing of the extracted DNA using Illumina technology by a sequencing service provider (e.g., Novogene).
3. **Quality Control:** Use FastQC on the Galaxy server to assess the quality of the sequencing data.
4. **Genome Assembly:** Perform *de novo* assembly of the sequencing reads using the Shovil (Spades) tool on the Galaxy server.
5. **Genome Annotation:**

- Predict structural and functional features of the assembled genome using the RAST server.
- Confirm annotations using BLASTP searches against existing databases.

6. **Genome Analysis:**
- Utilize the Proksee server for genome mapping and GC content analysis.
- Predict potential tRNA genes using tRNAscan-SE.
- Analyze the phage life cycle and other features using the Phage AI tool.

7. **Antibiotic Resistance and Virulence Screening:**
- Search for antibiotic resistance genes, virulence factors, and toxins within the phage genome using databases like CARD, ResFinder, and VFDB.

8. **Phage Naming and Comparison:**
- Name the isolated phage following Adriaenssens and Brister's methodology.

9. Perform a **comparative analysis** of gene arrangement among different phages using Easyfig software.

10. **Genome Similarity and Phylogenetic Analysis:** Determine genome-to-genome similarity and average nucleotide identity (ANI) using the VIRIDIC tool.

11. **Conduct a phylogenetic analysis** using VICTOR to understand evolutionary relationships with other known phages.

Note: This protocol provides a general framework for whole genome sequencing and analysis of isolated bacteriophages. Specific steps and tools may vary depending on the chosen platform and desired analysis depth.

Phage Preparation and Cocktail Formation

35 **1. Filtration and Centrifugation:**
- Filter the phage suspensions sequentially using an Amicon 100 kDa filter.
- Centrifuge the filtered suspensions at 5,000 ×g for 15 minutes.
- Sterilize the centrifuged suspensions using a 0.22 µm filter.

2. Phage Titer Determination:
- Determine the phage titer of each sterilized suspension using the DLA method.

3. Cocktail Formation:
- Combine equal volumes of each phage suspension, standardized by titer, in a sterile container.
- Thoroughly mix the combined suspensions to create the phage cocktail.

In Vivo Phage Therapy for MRSA Mastitis

36 **Chemicals and Reagents**
- Ketamine/xylazine mixture (9:1)
- Sterile PBS
- Ceftiofur sodium (5 mg/kg)

- MRSA strain (1.5 × 10^8 CFU/ml)
- Phage cocktail-1 (1 × 10^10 PFU/ml)

Equipment

- Syringes (27-gauge insulin syringe, 31-gauge insulin syringe)
- Homogenizer
- Centrifuge
- Microcentrifuge tubes
- BHI agar plates
- Incubator
- Pipettes
- Micropipettors
- Sterile containers
- Sterile filter paper (0.22 µm)

Additional Considerations

- Animal housing and care facilities
- Personnel trained in animal handling and experimental procedures
- Ethical approval for animal experiments
- Laboratory equipment and supplies for bacterial culture and phage titration
- Safety equipment (gloves, lab coat, safety glasses)

Procedure:

1. Animal Preparation:

- Procure 36 female BALB/c mice, aged and weight-matched.
- Ensure mice have a recent parturition history and are lactating (10-14 days postpartum).
- Acclimate mice for 24 hours.

2. Group Assignment:

- Randomly divide mice into six groups of six animals each:
- **Group 1 (Blank Control):** No treatment.
- **Group 2 (Negative Control):** MRSA infection, followed by sterile PBS injection.
- **Group 3 (Positive Control):** MRSA infection, followed by ceftiofur sodium injection (5 mg/kg).
- **Group 4 (Phage Therapy 1):** MRSA infection, followed by phage cocktail-1 injection (1 × 10^{10} PFU/mL).
- **Group 5 (Phage Therapy 2):** MRSA infection, followed by phage cocktail-2 injection (1 × 10^{9} PFU/mL).
- **Group 6 (Phage Control):** Phage cocktail-1 injection (1 × 10^{10} PFU/mL) without prior infection.

3. Mastitis Induction:

- Anesthetize mice with 100 µl of ketamine/xylazine mixture (9:1) via intraperitoneal injection.
- Make a small incision near the tip of each teat.

- Inject 25 µl of MRSA (1.5×10^8 CFU/mL) into the teat canals of the L4 and R4 mammary glands.

4. Treatment Administration:

- Four hours post-infection, inject 25 µl of the appropriate treatment into the infected mammary glands:
- **Group 1:** No treatment.
- **Group 2:** Sterile PBS.
- **Group 3:** Ceftiofur sodium.
- **Group 4:** Phage cocktail-1.
- **Group 5:** Phage cocktail-2.
- **Group 6:** Phage cocktail-1.

Blood Sample Collection and Analysis

37 **Equipment**

- Syringes (for anesthesia and blood collection)
- Needles
- Scalpel
- Microcentrifuge tubes
- Ice bucket
- Centrifuge

Chemicals and Reagents

- Ketamine/xylazine mixture (for anesthesia)
- Anticoagulant (e.g., EDTA) for blood collection tubes

Procedure:

1. Animal Euthanasia:

- At 24 and 48 hours post-treatment, anesthetize three mice per group with an intraperitoneal injection of ketamine/xylazine.
- Euthanize mice by cervical dislocation.

2. Blood Sample Collection:

- From two euthanized mice per group, collect 1 mL of blood via cardiac puncture.

3. Blood Sample Analysis:

- Transport blood samples to the laboratory.
- Perform complete blood counts (CBCs) on the blood samples.
- Measure the following parameters:
- White blood cells (WBC, 1000/µl)
- Red blood cells (RBC, ml/µl)
- Neutrophilic granulocytes (NE, 1000/µl)
- Hemoglobin (HGB, g/dl)
- Hematocrit (HCT, %)
- Platelet count (1000/µl)

Bacterial Colony Count and Phage Titer Determination

38 **Equipment**

- Homogenizer
- Centrifuge
- Microcentrifuge tubes
- BHI agar plates
- Incubator
- Pipettes
- Micropipettors
- Sterile containers
- Sterile filter paper (0.22 µm)

Chemicals and Reagents

- Sterile PBS
- 1x LB broth
- Soft agar
- Top agar
- *Staphylococcus aureus* ATCC 43300

Procedure:

1. Sample Preparation:

- Homogenize 100 mg of mammary gland tissue in 500 µL of sterile PBS.
- Centrifuge the homogenate at 15,000 rpm for 10 minutes at 4°C.
- Collect the supernatant and transfer it to a new microtube.

2. Serial Dilutions:

- Prepare serial dilutions (10^{-1} to 10^{-9}) of each sample in 1x LB broth.

3. Bacterial Colony Count:

- Plate 10 µL aliquots of dilutions 10^{-1} to 10^{-4} on BHI agar plates.
- Incubate plates at 37°C for 24 hours.
- Count the colony-forming units (CFU) on each plate.

4. Phage Titer Determination (for phage-treated groups):

- For groups 4, 5, and 6, determine the plaque-forming units (PFU) using the DLA technique.