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An optimized and High Yielding Protocol for Isolation and Amplification of Bacteriophages Against Methicillin-resistant Staphylococcus aureus (MRSA)

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Phage related protocols

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ABSTRACT

Bacteriophages are bacteria-specific viruses having great potential as therapeutic agents against multidrug-resistant strains/bacteria.Though "phage therapy" is a century-old concept, there is very limited progress on its therapeutic application due to the rapid expansion in antibiotics portfolios in the last few decades. However, the emergence of multidrug-resistant organisms in the recent past has brought back our attention to bacteriophages. The first step towards developing effective phage therapy against multidrug-resistant bacteria is isolation, amplification, and purification of specific bacteriophages. There are many reported protocols for the isolation of host-specific bacteriophages from the environment. However, most of them are complex, multistep, low-yielding, resource-intensive protocols, requiring elaborate laboratory setup. We have demonstrated a simple two-step, high-yielding protocol for isolating and amplifying bacteriophages against Methicillin-Resistant *Staphylococcus aureus* (MRSA). We have shown that mixing various environmental samples (i.e. sample pooling) and phage amplification at two different temperatures significantly enhance the yield of MRSA phages.

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KEYWORDS

Bacteriophage, methicillin-resistant Staphylococcus aureus (MRSA), Sample pooling, Phage yield, Environmental phages

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MATERIALS TEXT

Step 1:

Untreated sewage from a domestic sewage treatment plant
Untreated sewage from hospital sewage treatment plant
Chicken fecal samples from the poultry farm
Sterile saline (0.85%)
Sample collection bottles
50 mL centrifuge tube
Sterile 250 mL Filtration Unit
Sterile 250 ml conical flask
0.2 µm filtration membrane
Centrifuge
Sterile 250 ml reagent bottle
Laminar Air Flow
Sterile latex hand gloves

Step 2:

Sterile Tryptone Soy Broth (TSB)
Frozen/streaked culture of MRSA
Sterile 50 mL centrifuge tube
Incubator at 37 ^{0}C
Micropipette
Sterile micropipette tips

Step 3:

Mixed sample filtrate (from Step 1)
Sterile 2X TSB (HiMedia, India)
Sterile 50 mL glass conical flask (Borosil, India)
Overnight grown pure culture of MRSA (from Step 2)
Incubator at 37 ^{0}C
Micropipette
Sterile micropipette tips

Step 4:

Sterile Soft agar (TSB + 0.6% Agar)
SM Buffer
Overnight grown pure culture of MRSA (from Step 2)
Incubator at 37 ^{0}C
Tryptone soy agar plates
Sterile 5 ml / 15 ml centrifuge tubes
Manual colony counter (optional)
Micropipette
Sterile micropipette tips

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STEP 1: PREPARATION OF WATER SAMPLE FILTRATE FOR ISOLATION OF BACTERIOPHAGES (CRITICAL STEP)*

1 Collect untreated sewage water samples from the domestic sewage treatment plant and hospital wastewater treatment plant.

One can also use any alternative environmental sample for the isolation of specific phages. Keep the collected samples at 4 ^{0}C to avoid sample degradation.

2 Collect solid fecal samples from cages of chicken poultry farm and store them at 4°C until further processing.

4 °C

3 Measure 40 mL of each sewage sample and centrifuge them in 50 mL centrifuge tubes at 7000 rpm for 15 min.

40 mL **7000 rpm, 25°C**

Eppendorf™ 5810R Centrifuge
Centrifuge
Eppendorf **02-262-8187**

To avoid lab contamination with environmental strains, samples should be handled carefully with gloved hands

4 Filter the supernatant obtained through a 0.2 µm filter, and store the filtrate at 4 ^{0}C.

4 °C

One needs to be careful about spreading contamination from these sewage samples.

Weigh 10 g of dry poultry farm fecal sample and mix it with 100 mL of sterile saline in a sterile

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5 250 mL conical flask.

10 g **100 mL**

Samples should be handled carefully with all microbiological safety protocols.

6 Keep the suspension at 30 ^{0}C for 5 – 6 h at 120 rpm to detach the phages from solid particles.

30 °C **00:00:00** **6 hr**

7 Collect 40 mL of this sample and centrifuge it at 7000 rpm for 15 min.

40 mL **7000 rpm, 25°C**

Samples should be handled aseptically. After use, the centrifuge should be cleaned with suitable surface sterilization methods to prevent contamination.

8 Filter the supernatant obtained through a 0.2 µm filter, and store the filtrate at 4 ^{0}C.

4 °C

9 Mix all three filtrates (Domestic sewage, hospital wastewater, and poultry farm) in a sterile environment to make a single filtrate suspension (pooled sample) for isolation of MRSA-specific bacteriophages.

All three samples should be mixed in laminar airflow, and the suspension should be kept at 4 ^{0}C for further use.

STEP 2: GROWTH OF BACTERIAL HOST

10 Thaw the glycerol stock of MRSA stored at -80 $^{\circ}$C.

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Thawing should be performed by tapping to gradually increase *the temperature to avoid sudden heat shock.*

11 Inoculate 5 µL of the thawed culture in 20 mL of TSB in a 50 ml sterile conical flask and incubate for 24 h at 37 ^{0}C and 120 rpm.

5 µL **10 mL** **37 °C**

12 The grown culture was centrifuged at 10,000 rpm for 10 min and resuspended the pellet in 10 mL of fresh TSB. Use this suspension for the isolation and enrichment of MRSA-specific bacteriophages.

10 mL **10000 rpm, 25°C**

STEP 3: ENRICHMENT/AMPLIFICATION OF MRSA BACTERIOPHAGES

13 Mix 10 mL of pooled sample filtrate (obtained Step 1) and 10 mL of sterile 2X TSB in a 50 mL sterile glass conical flask.

10 mL

Some literature says the addition of 5 mM of $CaCl_2$ and $MgCl_2$ promotes phage adsorption on the host cell surface. In this case, the addition of $CaCl_2$ and $MgCl_2$ in TSB leads to precipitation of salts and should not be added if working with TSB.

14 Add 1 mL of an overnight grown pure culture of MRSA (obtained from Step-2) to the above suspension and mix gently. Incubate the suspension at 30 ^{0}C for 24 h at 85 rpm.

1 mL **30 °C**

A temperature of 30 ^{0}C is known to induce maximum adsorption of phages on the host cell surface. A low shaking speed at 85 rpm prevents the desorption of phages from the host cell surface while providing gentle mixing for bacterial growth.

15 Following incubation at 30 ^{0}C, Incubate the suspension at 4 ^{0}C for 24-48 hr.

4 °C

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16 After 24-48 hr, centrifuge the suspension at 14,000 rpm for 15 min and filter the supernatant through a sterile 0.2 µm syringe filter.

14000 rpm, 25°C

17 Quantify the titer of bacteriophages in the filtrate using the drop cast method (as described in step 4).

STEP 4: QUANTIFICATION OF BACTERIOPHAGES BY DROP CAST TEST

18 Quantification of bacteriophages in the filtrate obtained in any step of the experiment can be done using the drop cast method.

Chhibber S, Kaur P, Gondil VS (2018). Simple drop cast method for enumeration of bacteriophages.. Journal of virological methods. https://doi.org/10.1016/j.jviromet.2018.09.001

The method is called the drop cast method due to the spotting of drops on the lawn of the bacterial host and counting the plaque developed in the drop.

19 Take 100 µL of overnight grown pure culture of MRSA (from Step-2) and mix with 3 mL of molten soft agar (TSB + 0.6% Agar).

100 µL **3 mL**

20 Pour the suspension on prepared hard agar (TSB + 2% Agar) and allow it to solidify.

This step should be done in laminar airflow.

21 To quantify bacteriophages in the given filtrate, serially dilute the filtrate in SM buffer and spot 5 µL of each dilution on the bacterial lawn. Allow the plates to solidify completely.

5 µL

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22 Incubate the solidified plates overnight at 37 ^{0}C for the development of clear visible plaques.

37 °C

23 Calculate the phage titer (PFU/mL) by using the following formula:

$$\text{Number of PFU/mL} = \frac{\text{Number of plaques in a drop X Dilution factor}}{\text{Volume of drop spotted}}$$

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