APSi Alliance for Pathogen Surveillance Innovations – India

Surveillance of pathogens contributing to AMR in milk of dairy cattle

Council of Scientific and Industrial Research-National Chemical Laboratory (CSIR-NCL), Pune

Protocol handbook - 2024

Contents:

1. Introduction:

The presence of pathogens contributing to antimicrobial resistance (AMR) in biological samples such as dairy milk is of public health importance. Presence of these pathogens could be precipitated due to the misuse of antibiotics in dairy cattle and other factors. Studying the prevalence of bacterial pathogens and associated AMR genotypes from animal sources is hence important. The presence of AMR genotypes in milk microbiota is largely unknown in the Indian context. This protocol describes the use of 16S amplicon-based metagenomics for the identification of bacteria species.

2. Sample collection:

To collect a meaningful milk sample while ensuring cleanliness and avoiding contamination from external sources, follow these detailed steps:

1. Prepare yourself and the label:
    - Wash your hands thoroughly.
    - Wear new disposable gloves.
    - Use a waterproof marker to label the sample vial, recording the date, cow ID, and the specific quarter of udder (RF = right front, LF = left front, RR = right rear, LR = left rear).
2. Clean the Udder and Teats:
    - Brush off any loose manure, dirt, or bedding from the udder and teats.
    - Apply a germicidal teat dip and leave it on for 30 seconds. If the udder and teats are extremely dirty, wash and dry them thoroughly before applying the dip.
3. Dry the Teats:
    - Use a single-use paper or cloth towel to dry each teat, focusing on the teat end. Ensure no dip remains on the teat to avoid killing bacteria in the sample.
4. Pre-Sample Milk Disposal:
    - Discard the first 3 to 4 streams of milk onto the floor. This minimizes contamination from bacteria that may be present in the teat canal.
5. Disinfect the Teat Ends:
    - Vigorously scrub the teat ends for 10-15 seconds with a cotton ball or gauze pad soaked in 70% isopropyl alcohol, using as many as necessary until clean. Start with the far teats and move to the near ones, using a new swab for each teat to avoid re-contamination. Ensure the teats are not dripping with alcohol, as it can kill bacteria in your milk sample.
6. Collect the Milk Sample:
    - Open the sample vial just before collecting the milk to avoid contamination.

- Do not touch the inside of the vial or cap, or allow the teat end to touch the vial.
- Hold the vial at an angle to prevent dirt or hair from falling into it.
- Direct milk streams into the vial without touching the teat end, starting with the near teats and then the far ones. Fill the vial about 1/3 full to reduce contamination risk and prevent bursting when frozen.
- Close the vial immediately to make it airtight.

7. Store the Sample:
    - Place the sample vial on ice or in the refrigerator immediately.
    - Keep the samples cool until they are plated.
    - Freeze samples that will not be plated within 24 hours.

These steps are designed to ensure the milk sample is collected in a manner that minimizes contamination and preserves the integrity of the sample for analysis.

Alternate Sampling strategy:

If samples need to be transported at room temperature, GenePath Transport Medium (GTM) [catalogue number GPX-GTM-V2] can be used. The medium contains various lysis reagents that lyse the organisms in the sample, facilitating convenient transport or storage at room temperature.

Procedure:

1. Take a 15 ml centrifuge tube and add 500 µl of GTM.
2. Add an equal volume of milk to the GTM solution.
3. Invert the tube two to three times to mix the solution properly.

The samples can then be stored at room temperature for up to one week.

3. Sample storage:

When handling milk samples, optimal storage conditions are crucial for preserving their quality and integrity before analysis. Here are the recommended guidelines for storing milk samples, depending on when you plan to get them plated:

1. Room Temperature:
    - Store the samples at room temperature only if they will be plated within less than 1 hour. Room temperature storage is not suitable for longer periods due to the risk of bacterial growth and degradation of the sample.
2. Refrigerator Temperature:
    - If immediate plating is not possible, store the samples in a refrigerator. This is suitable for samples that will be plated within more than 1 hour but less than 2 days. Refrigeration slows down bacterial growth, helping preserve the sample's integrity for a short period.

3. Freezer Temperature:
    - For samples that cannot be plated within 2 days, freezing is recommended. Samples can be stored in the freezer for more than 2 days but should be used within less than 60 days. Freezing preserves samples for extended periods by significantly slowing or stopping bacterial growth.

Organizing Frozen Samples:

- If storing multiple samples in the freezer for extended periods, consider grouping them by week. Place them in a plastic bag or box labelled with the collection dates. This organizational method facilitates easy retrieval of specific samples when needed, ensuring efficient sample management and analysis.

These storage guidelines ensure that milk samples remain viable for analysis, reducing the risk of contamination and ensuring accurate results.

4. Milk Culturing:

The outlined procedures are essential steps for culturing samples, specifically milk samples for microbiological analysis, ensuring accurate and reliable results:

Sample Preparation

1. Storage: If immediate plating is not possible, refrigerate or freeze the sample. This prevents sample degradation.
2. Thawing: For frozen samples, allow complete thawing in the refrigerator before proceeding.
3. Mixing: Ensure the sample vial lid is sealed. Gently invert the sample vial around 15 times to mix.

Plating Procedure

1. Personal Preparation: Wash hands and wear new disposable gloves to maintain hygiene.
2. Plate Labeling: On the bottom (reverse side) of the plate, label each sample with cow ID, quarter, and date. Ensure the plate is turned upside down for this step.
3. Swab and Plate Preparation: Use a new sterile cotton swab and a new plate for each sample to avoid contamination and ensure reliability.
4. Avoiding Contamination: Prevent touching the swab's cotton end or the plate with fingers or any surface. Open the swab package in a way that keeps the cotton end covered.
5. Sample Collection: Saturate the sterile cotton swab with the milk sample by rolling it in the sample for 8-10 seconds. Ensure full saturation.
6. Handling Clumps: Remove any milk clumps from the swab to prevent misinterpretation of results as these are not indicative of bacterial growth.

7. Application on Media: Using the saturated swab, streak the sample across the entire surface of the media section. Re-dip the swab in milk between streaking different sections.
8. Sealing and Freezing Sample: After plating, immediately replace the lid on both the plate and the milk sample. Freeze the milk sample for potential future testing.
9. Incubation: Place the plated samples in a 37° C incubator for up to 48 hours, with the media facing down to prevent condensation interference.
10. First Reading: After 18 to 24 hours, conduct the first reading of the plate.
11. Maintaining Cleanliness: Ensure cleanliness throughout the process to avoid contamination from external sources such as manure or dust. Disinfect work surfaces and manage spills promptly.

These steps are designed to maximize the accuracy of bacterial growth detection and analysis, crucial for diagnosing and managing bacterial infections in dairy cows or for ensuring the safety and quality of milk products.

5. Culture Medium:

The media components listed are essential for culturing milk samples to identify specific bacteria and assess their antibiotic susceptibility. Each type of agar or broth serves a unique purpose, targeting different bacterial species or providing a general growth environment. Here's a brief overview of each media component and its application:

1. **MacConkey Agar**: This medium is selective for Gram-negative bacteria and is commonly used to isolate Escherichia coli (E. coli). The agar differentiates lactose fermenters, which produce pink colonies, from non-lactose fermenters.
2. **Gelatin Mannitol Salt Agar**: This medium is selective for Staphylococcus species due to its high salt concentration, which inhibits the growth of most other bacteria. Mannitol fermentation by Staphylococcus aureus results in yellow colonies, making it easy to differentiate from other staphylococci.
3. **Pseudomonas Agar**: Specifically designed for the isolation of Pseudomonas species, this medium supports the growth of Pseudomonas by providing essential nutrients and may include selective agents to differentiate various Pseudomonas species based on pigment production.
4. **BETA-SSA Agar** (Blood Erythritol Tetracycline Agar): Primarily used for the isolation and identification of Streptococcus species. This medium can differentiate Streptococcus species based on their hemolytic properties and other biochemical characteristics.
5. **Muller Hinton Agar**: This is the standard medium used for antibiotic susceptibility testing. It provides a uniform testing environment that allows the assessment of how different bacteria respond to antibiotics, thereby determining the most effective treatment for bacterial infections.

6. **Nutrient Broth**: A non-selective, liquid medium used for the preservation of cultures. Nutrient broth supports the growth of a wide range of bacteria, making it ideal for maintaining and propagating bacterial cultures before or after specific tests are conducted.

These media are crucial tools in microbiological analysis, allowing for the precise identification and characterization of bacteria present in milk samples. By utilizing these different media, microbiologists can detect harmful pathogens, ensure the safety and quality of dairy products, and guide effective treatment for bacterial infections.

6. DNA extraction: (SPINeasy® DNA Kit for Tissue & Bacteria- Cat. No. 6532050)

Preparation of Gram-Positive Bacteria Pre-Treatment Buffer:

Prepare a buffer containing:

20 mM Tris-HCl, pH 8.0

2 mM EDTA, pH 8.0

1.2% Triton® X-100

20 mg/mL lysozyme

Store the buffer at 4°C after adding lysozyme.

Pre-Treatment Steps:

a. Harvest cells by centrifugation at 10,000 x g for 3 minutes. Discard the supernatant.

b. Resuspend the bacterial pellet in 200 µL of the prepared gram-positive bacteria pre-treatment buffer.

c. Incubate for 30 minutes at 37°C.

DNA Extraction Steps:

a. Add 800 µL of Lysis Buffer GD to the bacterial suspension and mix thoroughly. Transfer this mixture to a vial of Lysing Matrix A.

b. Homogenize the mixture using a FastPrep® Instrument for 15 seconds at a speed setting of 4.0 m/s. Alternatively, vortex the samples at maximum speed for 20 minutes for animal tissues and bacteria or 10 minutes for blood and cultured cells. Use an adapter to hold the vials during vortexing, especially for processing multiple samples simultaneously.

c. Centrifuge the homogenized mixture at 14,000 x g for 10 minutes. If 14,000 x g is not feasible, centrifuge at the maximum speed for all steps.

d. Transfer 750 µL of the supernatant to a Column GD with a collection tube.

e. Centrifuge at 14,000 x g for 1 minute. Discard the flow-through and reuse the collection tube.

f. Add 500 µL of Wash Buffer GD1 to the column.

g. Centrifuge at 14,000 x g for 1 minute. Discard the flow-through and reuse the collection tube.

h. Add 750 µL of Wash Buffer GD2 to the column and incubate at room temperature for 1 minute.

i. Centrifuge at 14,000 x g for 1 minute. Discard the flow-through and reuse the collection tube.

j. Centrifuge at 14,000 x g for an additional 1 minute to dry the column.

k. Optionally, incubate at 55°C for 3 – 5 minutes to completely dry the column.

l. Remove the collection tube and place the column onto a clean 1.5 mL microcentrifuge tube.

m. Add 100 µL of Elution Buffer GD to the center of the membrane. Incubate at room temperature for 1 minute. For samples with low DNA content, reducing the elution volume to 50 µL may increase the concentration of eluted DNA.

n. Centrifuge at 8,000 x g for 1 – 2 minutes to elute DNA.

o. The eluted genomic DNA will be collected in the microcentrifuge tube.

7. Oxford Nanopore Technology (ONT) sequencing: (RAPID SEQUENCING DNA V14 - BARCODING (SQK-RBK114.24 OR SQK-RBK114.96)

To prepare the DNA in Nuclease-free water and barcode it for sequencing, follow these steps:

1. Transfer 200 ng of genomic DNA per sample into 0.2 ml thin-walled PCR tubes or an Eppendorf twin.tec® PCR plate 96 LoBind.
2. Adjust the volume of each sample to 10 µl with Nuclease-free water.
3. Pipette mix the tubes for 10-15 times to avoid unwanted shearing.
4. Spin down briefly in a microfuge.
5. In the 0.2 ml thin-walled PCR tubes or an Eppendorf twin.tec® PCR plate 96 LoBind, mix the following:
    - 10 µl Template DNA (200 ng from the previous step)
    - 1.5 µl Rapid Barcodes (RB01-24 or RB01-96, one for each sample)
6. Ensure the components are thoroughly mixed by pipetting and spin down briefly.
7. Incubate the tubes or plate at 30°C for 2 minutes and then at 80°C for 2 minutes. Briefly put the tubes or plate on ice to cool.
8. Spin down the tubes or plate to collect the liquid at the bottom.
9. Pool all barcoded samples in a clean 2 ml Eppendorf DNA LoBind tube, noting the total volume.
10. Resuspend the AMPure XP Beads (AXP) by vortexing.
11. Add an equal volume of resuspended AMPure XP Beads (AXP) to the entire pooled barcoded sample, and mix by flicking the tube.

- Volume of AMPure XP Beads (AXP) added:
    - Volume per sample: 11.5 µl
    - For 4 samples: 46 µl
    - For 12 samples: 138 µl
    - For 24 samples: 276 µl
    - For 48 samples: 552 µl
    - For 96 samples: 1,000 µl

12. Incubate on a Hula mixer (rotator mixer) for 10 minutes at room temperature (RT).
13. Prepare at least 2 ml of fresh 80% ethanol in Nuclease-free water.
14. Spin down the sample and pellet on a magnet. Keep the tube on the magnet, and pipette off the supernatant.
15. Keep the tube on the magnet and wash the beads with 1 ml of freshly prepared 80% ethanol without disturbing the pellet. Remove the ethanol using a pipette and discard. Repeat this step.
16. Briefly spin down and place the tube back on the magnet. Pipette off any residual ethanol. Allow to dry for 30 seconds, but do not dry the pellet to the point of cracking.

To complete the library preparation and prepare the flow cell for sequencing, follow these steps:

Resuspension and Elution:

1. Remove the tube from the magnetic rack and resuspend the pellet in 15 µl Elution Buffer (EB) per 24 barcodes used.
    - Volume of Elution Buffer (EB):
        - For 24 barcodes: 15 µl
        - For 48 barcodes: 30 µl
        - For 72 barcodes: 45 µl
        - For 96 barcodes: 60 µl
2. Incubate for 10 minutes at room temperature (RT).
3. Pellet the beads on a magnet until the eluate is clear and colorless, for at least 1 minute.
4. Remove and retain the full volume of eluate into a clean 1.5 ml Eppendorf DNA LoBind tube.
5. Dispose of the pelleted beads.

Quantification:

1. Quantify 1 µl of eluted sample using a Qubit fluorometer.

2. Transfer 11 µl of the sample into a clean 1.5 ml Eppendorf DNA LoBind tube.

Adapter Dilution and Ligation:

1. Dilute the Rapid Adapter (RA) as follows and pipette mix:
    - 1.5 µl Rapid Adapter (RA)
    - 3.5 µl Adapter Buffer (ADB)
2. Add 1 µl of the diluted Rapid Adapter (RA) to the barcoded DNA.
3. Mix gently by flicking the tube, and spin down.
4. Incubate the reaction for 5 minutes at RT.

Flow Cell Priming:

1. Thaw the Sequencing Buffer (SB), Library Beads (LIB) or Library Solution (LIS, if using), Flow Cell Tether (FCT) and Flow Cell Flush (FCF) at RT before mixing by vortexing. Then spin down and store on ice.
2. For optimal sequencing performance and improved output on MinION R10.4.1 flow cells (FLO-MIN114), add Bovine Serum Albumin (BSA) to the flow cell priming mix at a final concentration of 0.2 mg/ml.
3. To prepare the flow cell priming mix with BSA, combine the following reagents in a fresh 1.5 ml Eppendorf DNA LoBind tube. Mix by inverting the tube and pipette mix at RT:
    - 1,170 µl Flow Cell Flush (FCF)
    - 5 µl Bovine Serum Albumin (BSA) at 50 mg/ml
    - 30 µl Flow Cell Tether (FCT)
    - 1,205 µl Final total volume in tube

After completing these steps, the prepared library can be loaded into the flow cell for sequencing. Store the library on ice until ready to load.

To load the MinION or GridION device and prepare the flow cell for sequencing, follow these steps:

Flow Cell Preparation:

1. Open the MinION or GridION device lid and slide the flow cell under the clip. Press down firmly on the flow cell to ensure correct thermal and electrical contact.
2. Slide the flow cell priming port cover clockwise to open the priming port.

Bubble Removal:

1. After opening the priming port, check for a small air bubble under the cover. Draw back a small volume to remove any bubbles:
    - Set a P1000 pipette to 200 µl.

- Insert the tip into the priming port.
- Turn the wheel until the dial shows 220-230 µl, to draw back 20-30 µl, or until you can see a small volume of buffer entering the pipette tip.
- Note: Visually check that there is continuous buffer from the priming port across the sensor array.

Flow Cell Priming:

1. Load 800 µl of the priming mix into the flow cell via the priming port, avoiding the introduction of air bubbles. Wait for five minutes. During this time, prepare the library for loading.

Library Preparation:

1. Thoroughly mix the contents of the Library Beads (LIB) by pipetting.
2. In a new 1.5 ml Eppendorf DNA LoBind tube, prepare the library for loading as follows:
    - 37.5 µl Sequencing Buffer (SB)
    - 25.5 µl Library Beads (LIB) mixed immediately before use, or Library Solution (LIS), if using
    - 12 µl DNA library

Completing Flow Cell Priming:

1. Gently lift the SpotON sample port cover to make the SpotON sample port accessible.
2. Load 200 µl of the priming mix into the flow cell priming port (not the SpotON sample port), avoiding the introduction of air bubbles.
3. Mix the prepared library gently by pipetting up and down just prior to loading.
4. Add 75 µl of the prepared library to the flow cell via the SpotON sample port in a dropwise fashion. Ensure each drop flows into the port before adding the next.
5. Gently replace the SpotON sample port cover, making sure the bung enters the SpotON port and close the priming port.

Final Step:

- Install the light shield on your flow cell as soon as the library has been loaded for optimal sequencing output.

Following these steps will ensure proper loading of the library onto the flow cell for sequencing.

8. Data analysis

Bacterial assembly and annotation workflow

(https://github.com/epi2me-labs/wf-bacterial-genomes.git)

Assembly, variant calling, and annotation of bacterial genomes.

This workflow is primarily used to assemble genomes from bacterial reads and provide information on features of interest within those assemblies through annotations.

The workflow can provide additional information about the assembly, such as antimicrobial resistance (AMR) analysis and sequence typing through an optional --isolates mode.

In brief, this workflow will perform the following:

- De novo (or reference-based) assembly of bacterial genomes
- Annotation of regions of interest within the assembly
- Species identification and sequence typing (--isolates mode only)
- Identify genes and SNVs associated with AMR (--isolates mode only)

Install and run

These are instructions to install and run the workflow on command line. You can also access the workflow via the EPI2ME application.

The workflow uses Nextflow to manage compute and software resources, therefore nextflow will need to be installed before attempting to run the workflow.

The workflow can currently be run using either Docker or Singularity to provide isolation of the required software. Both methods are automated out-of-the-box provided either docker or singularity is installed. This is controlled by the -profile parameter as exemplified below.

It is not required to clone or download the git repository in order to run the workflow. More information on running EPI2ME workflows can be found on our website.

The following command can be used to obtain the workflow. This will pull the repository in to the assets folder of nextflow and provide a list of all parameters available for the workflow as well as an example command:

nextflow run epi2me-labs/wf-bacterial-genomes –-help

A demo dataset is provided for testing of the workflow. It can be downloaded using:

Wget https://ont-exd-int-s3-euwst1-epi2me-labs.s3.amazonaws.com/wf-bacterial-genomes/wf-bacterial-genomes-demo.tar.gz

tar -xzvf wf-bacterial-genomes-demo.tar.gz

The workflow can be run with the demo data using:

nextflow run epi2me-labs/wf-bacterial-genomes --fastq wf-bacterial-genomes-demo/isolates_fastq –isolates --sample_sheet wf-bacterial-genomes-demo/isolates_sample_sheet.csv -profile standard

For further information about running a workflow on the cmd line see https://labs.epi2me.io/wfquickstart/

9. References:

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3. https://community.nanoporetech.com/docs/prepare/library_prep_protocols/rapid-sequencing-gdna-barcoding-sqk-rbk114/v/rbk_9176_v114_revm_27nov2022
4. https://github.com/epi2me-labs/wf-bacterial-genomes

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