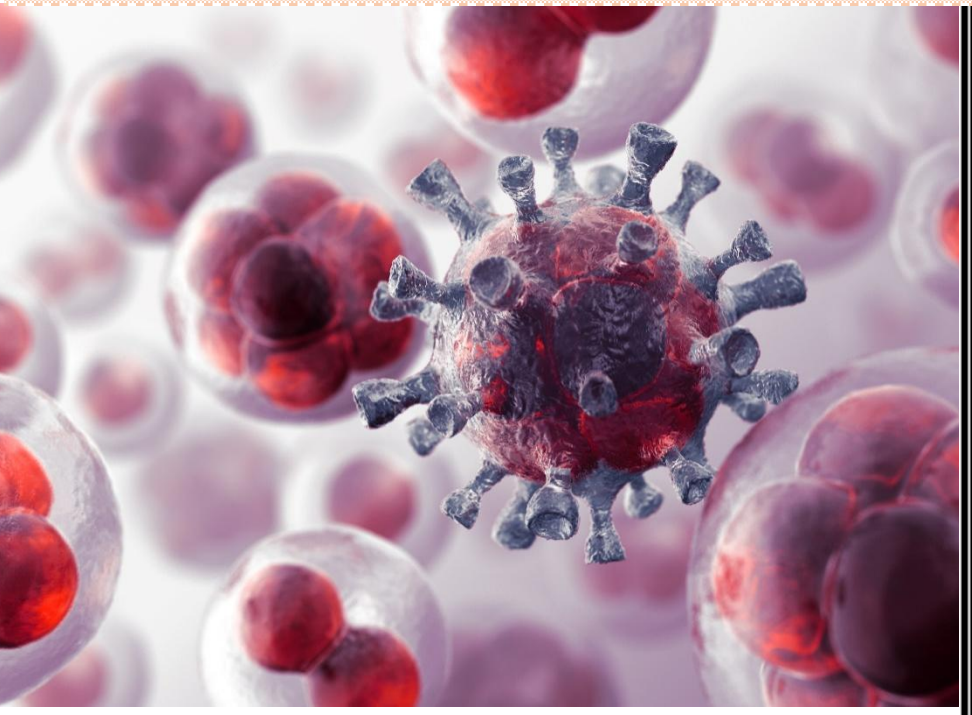
APSi Alliance for Pathogen Surveillance Innovations – India

Detection of pathogens contributing to antimicrobial resistance in clinical samples



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Protocol handbook - 2024

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ABOUT

This standard operating procedure (SOP) outlines the end-end protocol for whole genome sequencing (WGS) of bacterial organisms using the Oxford Nanopore Technology (ONT) platform. The document includes detailed procedures for library preparation and an extensive computational pipeline to identify antimicrobial resistance genes (ARGs) and mobile genetic elements (MGEs) from assembled genomes. This SOP applies to all laboratories conducting WGS of both Gram-positive and Gram-negative bacteria.

Key Interventions and Applications

Implementing this SOP allows for various critical interventions that can support clinicians and genomic researchers by providing comprehensive data from bacterial WGS:

- **Regular Surveillance for Priority Pathogens**: Enhances ongoing monitoring and understanding of priority pathogen. **Suspected Outbreak**: Facilitates the investigation and confirmation of outbreak scenarios
- **Suspected Nosocomial Infection**: Aids in identifying hospital-acquired infections
- **Inability to Detect the Infectious Agent by Traditional Methods**: Provides an alternative when standard tests fail
- **Possibility of a Coinfection**: Detects coinfections that may be missed by traditional methods
- **Discrepancy Between Predicted Sensitivity by Laboratory Tests and Patient Response**: Identifies cases where lab tests and patient outcomes differ

These can be achieved using platforms like Illumina and ONT, which provide high-resolution data for accurate pathogen identification and resistance gene detection Additionally, using kit-based detection methods can further aid in the rapid identification and characterization of pathogens in clinical and environmental samples.

Standard protocol for identifying ARGs and MGEs using WGS of pathogens isolated from clinical samples

1. Protocol for clinical sample collection, antibiotic susceptibility testing and DNA extraction

1.1. Sample collection and processing from various clinical specimens (as per ICMR guidelines)

1.1.1 Sample Collection & Processing from Blood

A. Equipment required

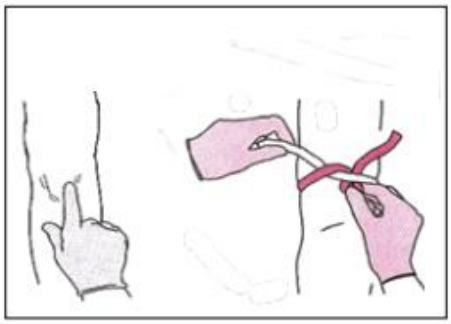
- BacT/ALERT 3D
- Incubator

B. Consumables required

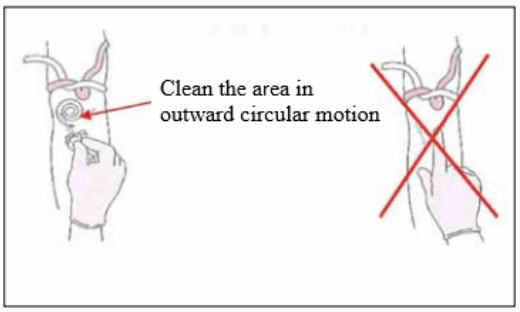
- BacT/ALERT PF Plus bottle
- Media (Blood agar, MacConkey agar, Nutrient agar, XLD agar, Hichrome *E. coli* agar, Glycerol broth)
- Standard antibiotic impregnated discs (Himedia)
- Inoculation loop (10 µL and 1 µL)
- Sterile petri dish (60mm, 75mm and 150mm)
- Sterile cotton swab
- Sterile 0.45% normal saline
- Sterile disposable syringes
- 70% isopropyl alcohol or ethyl alcohol
- 10% w/v povidone iodine

C. Collection of blood

Blood is to be collected following proper aseptic procedure. The site of collection should be thoroughly cleaned with proper antiseptic (70% isopropyl or ethyl alcohol first, followed by 10% w/v povidone iodine, and again followed by 70% isopropyl or ethyl alcohol). The direction of cleaning the site of puncture is a circular manner starting from the centre towards the outward direction. It is allowed to dry at least for 30 seconds between application. Sterile gloves should be worn when performing the procedure.



a. Select the site
b. Clean the site
c. Apply tourniquet 3-4 inch above the site



d. Use sterile gloves
e. Cotton-soaked spirit/70% isopropyl alcohol
f. Clean in outward circular motion
g. Repeat 2-3 times
h. Povidine iodine/Tincture iodine/1-2% chlorhexidine
i. Clean & wait for 3 full minutes
j. Do not palpate area with same glove

k. Instruct to clench fist
l. Release tourniquet
m. Withdraw needle
n. Flip off blood culture bottle lid
o. Gently inoculate 6-10 ml in a pediatric culture bottle

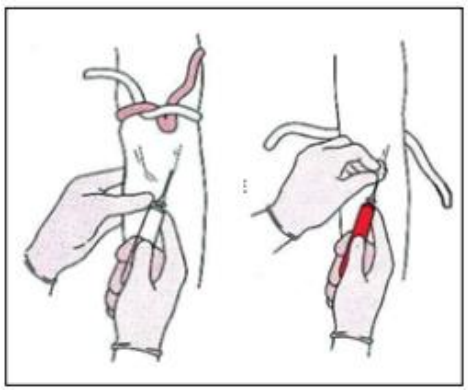


Figure 1: Aseptic method of blood collection for blood culture

D. Preparation of the blood culture bottle

Label the bottles with the patient's name and the date and time of draw. The septum of the bottle is wiped with an alcohol-soaked swab before inoculation.

Table 1: Recommended total volume and numbers of blood cultures:

Age & body weight	**Amount (divided between 2 blood cultures)**	**Remarks**
Neonates to 1 year (<4 kg)	0.5 to 1.5 ml	At least 1 ml Two separate venipunctures are generally not possible
Children (< 40 kg)	10 to 20 ml	Blood culture volumes will be limited to <1% of total blood volume (usually about 0.7 ml/kg). e.g. total sample limit would be 7 ml for a 10 kg patient and 28 ml for a 40 kg patient.
Children (>40 kg)	30 to 40 ml	At least 10-20 ml of blood

- Anaerobic blood cultures can be taken on request by treating clinician.
- Pediatric patient: 6-10 ml, divided between two blood cultures.
- Initially obtain three blood culture sets within a 30-minute period before administration of empiric antimicrobial agents from patients presenting with possible infective endocarditis. If those sets are negative at 24 hours, obtain two more sets of cultures, for a total of five sets overall.

Figure 2: BacT/ALERT PF Plus Bottle

E. Timing of blood collection

Blood should be drawn preferably before starting antibiotic therapy. If antibiotic therapy has already started, blood should be taken just before the start of the next dose.

Although drawing blood cultures before or during the fever spike is optimal for recovery, volume is more important than timing in the detection of agents of septicaemia. Thoroughly mix bottles to avoid clotting.

After phlebotomy, remove residual tincture of iodine from the patient's skin by cleansing with alcohol to avoid skin irritation.

F. Inoculation of blood into bottle

For conventional blood culture method, blood culture for bacterial infections will be carried out in two bottles containing 50 ml each of Brain Heart Infusion (BHI). After removing the kraft paper, inoculate into the blood culture bottles.

For the automated blood culture detection, the blood samples for bacterial infection will be inoculated into two BacT/Alert PF Plus culture bottles.

G. Transportation of blood culture bottles

All inoculated blood culture bottles should be transported at room temperature to the laboratory. In case of delay, the bottles should not be refrigerated. Instead, the bottles should be kept in an incubator at 35 $\pm$ 2 ºC, if available. Otherwise, leave the bottle at room temperature.

H. Processing of blood cultures

- For conventional blood culture method, incubate at 37°C and examine daily for 7 days for evidence of growth, indicated by turbidity, hemolysis, gas production, discrete colonies, or a combination of these.
- For the automated blood culture detection, BacT/Alert PF Plus culture bottles should be loaded into BacT/Alert instruments. Once the bottle is loaded in the BacT/ALERT, the sensor will be continuously analyzed every 10 minutes. Following a 5-day incubation period, any bottles flagged as positive by the BacT/Alert system undergo subculture and interpretation based on standard protocols.

1) Safety measures:

- Keep the culture bottles within a biosafety cabinet or behind a shield, or wear a face mask.
- Always wear gloves, because blood cultures contain material from patients who may harbor blood-borne pathogens.
- Use needleless transfer devices or safety needles, and never recap them.
- Dispose off needles and syringes in puncture-proof container.

2) For conventional system, incubate blood cultures for the predetermined period at 35 ± 2 ºC (usually 5 days, unless quality monitors indicate less time).
3) Examine the cultures at least daily by visual inspection for conventional system. For visual inspection, observe for hemolysis, turbidity, gas production, pellicle formation, "puffballs," and clotting, which are indicative of microbial growth.

Figure 3: BHI showing microbial growth

4) For manual broth systems, perform at least one blind subculture on blood agar and McConkey agar (MAC), from visually negative bottles. Perform blind subculture after overnight incubation, on alternate days.
   For automated system like BacT/Alert 3D, there is no need for blind subcultures. The equipment will signal for any growth or no growth by the end of 5th day of incubation.
5) Discard positive and negative bottles safely after autoclaving at 121ºC for 30 minutes. After autoclaving, open the automated system bottles with an opener and discard the inoculated medium in a designated shank for biomedical waste. For manual systems, open the screw-capped bottles, and discard the cultured media.

1.1.2 Sample Collection & Processing From Stool Samples

A. <u>Sample collection</u>

Stool samples are collected in a sterile wide mouth container with screw cap accompanied with a small scoop.

Figure 4: Sterile screw cap container with a small scoop

B. <u>Culture and Isolation</u>

- Faecal samples can be inoculated on the following media: XLD agar, MacConkey agar and HiChrome *E coli* media, using a sterile inoculating loop
- Place a loopful of the specimen over a small area of each plate, then flame the loop, and streak from the inoculated area over the entire plate.

1.1.3 Sample Collection & Processing From Urine Samples

A. Sample collection (Midstream clean catch urine):

- Preliminary cleaning of external genitalia with soap and water. Antiseptics should not be used.
- Voiding the first portion of urine.
- First voided morning specimen be collected.
- If not possible, the urine can be collected during the day, preferably 4 hours after the last void, keeping in mind that the counts maybe lower, yet significant.
- Collected in a sterile, wide mouth, screw capped container – strictly opened just before collection and closed immediately.

B. Culture and Isolation:

- Urine samples can be inoculated on the following media: Blood agar, MacConkey agar and HiChrome *E coli* media, using a sterile inoculating loop

- Place a loopful of the specimen over a small area of each plate, then flame the loop, and streak from the inoculated area over the entire plate.
- To evaluate the clinical significance of a growth in urine culture, a 1µl calibrated loop is used, that is, 10^5 CFU/mL.

C. Results:

- After overnight incubation, count the number of colonies manually on each plate and multiply the number of colonies counted by 10 for undiluted urine.
- This gives total number of viable bacteria present in 1.0 ml undiluted urine and express as CFU/ml of urine.

D. Interpretation of counts:

The significance of a positive urine culture is most reliably assessed in terms of the number of colony forming units (viable bacteria) present in the urine. The following is offered as a guide for midstream clean catch urine.

Table 2: Interpretation of counts

Number of colonies	**Significance**	**Interpretation**
<1000 CFU/ml	Insignificant (Bacteriuria)	UTI-unlikely
1000- 100,000 CFU/ml	Probably Significant (Bacteriuria)	UTI Probable
>100,000 CFU /ml	Significant (Bacteriuria)	UTI certain

1.1.4 Sample Collection & Processing From Ocular surfaces

To detect pathogens contributing to AMR in ocular samples, clinical samples from patients with ocular illnesses can be assessed. Samples from patients who are advised microbiological investigations and treatment by the ophthalmologists can be included. The common ocular conditions included for investigations may be corneal ulcers, endophthalmitis, dacryocystitis, conjunctivitis, scleritis, canaliculitis, uveitis etc. Type of clinical samples include:

- Corneal scrapings
- Corneal buttons (from recipient)
- Vitreous/aqueous aspirates

- Conjunctival swabs
- Donor sclero-corneal rims
- Iris
- Intraocular lens explant
- Contact lens

Culture methods: Most samples are processed for the growth of bacteria and fungi. Using the surgical blade # 15 the corneal scrapings are directly inoculated on the surface of solid media (blood agar, chocolate agar, Sabouraud dextrose agar, potato dextrose agar) in multiple `C' shaped streaks all over the plate. AC/Vitreous samples are inoculated by placing the drops on solid media surface and by directly injecting into liquid media. The media included are Blood agar (2- aerobic, anaerobic) (Himedia, Catalog:M073-500G), Chocolate agar (Himedia, Catalog: M073-500G), Brain-heart-infusion broth (Himedia, Catalog:M210-500G), Robertson's cooked meat broth (Himedia, Catalog: M149-500G), Thioglycollate broth (Himedia, Catalog:M979-500G), Sabouraud dextrose agar (Himedia, Catalog:GM096-500G), and Potato dextrose agar (Himedia, Catalog:GM033-500G). Colonies growing on the site of inoculum only are considered for further processing and identification. Colonies outside the inoculum mark are considered as contaminants. Colonies are first noted for their number, colour, and size. Any haemolysis (blood agar) or discoloration surrounding the colonies is also noted. A preliminary identification is made based on colony morphology and grams stain. The further identification of the bacteria is done by Vitek II compact automated identification system. Filamentous fungal species are identified based on rate of growth, colony morphology (colour, texture, consistency) and pattern of sporulation. The type of spores is studied by examining a lactophenol cotton blue preparation of the colony.

1.2. Antimicrobial Susceptibility Testing

1.2.1. Antimicrobial Susceptibility Testing using the Kirby-Bauer Disk diffusion method

The Kirby Bauer method of antibiotic susceptibility testing uses sterile filter paper discs that are impregnated with standard concentration of antibiotics, as laid down by Clinical and Laboratory Standards Institute (CLSI) guidelines, that tests the sensitivity of that particular antibiotic to the test organisms.

1) Preparation of media: The recommended media is Müeller Hinton agar (MHA) (Himedia, Catalog:M173-500G). Fresh plates are to be used the same day or stored in a refrigerator (2-8 ºC); if refrigerated, they will be wrapped in plastic to minimize evaporation. Just before use, if excess moisture is visible on the surface, plates will be placed in an incubator (35ºC) or, with lids ajar, in a laminar-flow hood at room temperature until the moisture evaporates (usually 10 to 30 minutes).
2) Preparation of inoculum: Prepare a saline suspension of the isolate from an overnight incubated agar plate (use a non-selective medium, such as nutrient agar) to obtain 0.5 McFarland turbidity (that is equivalent to 1.5 x 10^8 cfu/ ml of *E. coli* ®25922)

   With a sterile straight wire, touch the top of each of four to five colonies of the same morphological type, and inoculate onto the broth. Incubate the tube at 37ºC for 15 minutes or longer till turbidity of 0.5 McFarland. If turbidity is higher than 0.5 McFarland, sterile normal saline can be used to adjust turbidity to exactly 0.5 McFarland.
3) Inoculating test plates: MHA plate can be inoculated within 15 minutes after the inoculum has been adjusted. Dip a sterile cotton swab into the suspension, rotate it several times, and gently press onto the inside wall of the tube above the fluid level to remove excess inoculum from the swab. The swab should then be streaked over the entire surface to the agar plate three times, with the plate rotated approximately 60º each time to ensure even distribution of the inoculum. A final sweep of the swab will be made around the agar rim. The lid may be left ajar for 3 to 5 minutes but no longer than 15 minutes to allow any excess surface moisture to be absorbed before the drug-impregnated discs are applied.
4) Application of antimicrobial discs to an agar plate: Ideally, this will be done within 15 minutes of inoculation of plates. The selected antimicrobial discs will be dispensed evenly onto the agar plate with the help of a forceps/sterile needle/surgical blade. Flame the tips of the applicator intermittently. Each disc must be pressed down to ensure complete contact with the agar surface.

   Ordinarily, no more than 12 discs are applied on a 150 mm plate or 5 discs on a 100 mm plate, keeping at least a distance of 24 mm between discs. Dispensing too near to the edge of the plate should be avoided. Because some

of the drugs diffuse instantaneously, a disc will not be relocated once it has come in contact with the agar surface.

It is advisable to place discs that give predictably small zones like aminoglycosides, next to those discs that give larger zones like cephalosporins.

Disc containers are to be removed from the refrigerator or freezer one to two hours before use, so they may equilibrate to room temperature before opening. This procedure minimizes the amount of condensation that occurs when warm air contacts cold disks.

Note: *Sealed packages of the disks that contain drugs from the β-lactam class are to be stored frozen, except for a small working supply, which may be refrigerated for one week at most. Some labile agents (e.g. imipenem, cefaclor, and clavulanic acid combinations) may retain greater stability if stored frozen until the day of use.*

- Incubation: No longer than 15 minutes after discs are applied, the plates will then have to be inverted and incubated at 37ºC.
- Interpretation and reporting of results: Each plate should be examined after overnight incubation (16-8 hours), for confluent growth and circular zones of inhibition. The diameters of the zones of complete inhibition, including the diameter of the disc, will require to be measured to the nearest whole millimeter (mm) with a ruler. With unsupplemented MHA, the measuring device should be held on the back of the inverted petri dish, which is illuminated with reflected light located a few inches above a black, non-reflecting background.

  Zone margin is considered to be the area showing no obvious visible growth detectable with the unaided eye. Faint growth of tiny colonies visible only by lens can be ignored. Zone sizes have to be measured from the upper inoculated surface of opaque media like MHA with added blood, illuminated with reflected light, with the cover removed. In case of presence of discrete colonies within clear zone of inhibition, repeat test with a subculture of a single colony/pure culture from the primary culture plate. If discrete colonies still appear, inner colony free zone size are to be measured.

  With trimethoprim, the sulfonamides, and combinations of the two agents, antagonists in the medium may allow some minimal growth; therefore, the zone

diameter will be measured at the obvious margin, and slight growth (20% or less of the lawn of growth) is disregarded.

- Storage of antimicrobial discs: Cartridges containing commercially prepared paper disks specifically for susceptibility testing are generally packaged to ensure appropriate anhydrous conditions. Discs are to be stored at 8°C or below, or frozen at -14°C or below, in a non-frost-free freezer until needed. Sealed packages of disks that contain drugs from the ß-lactam class are to be stored frozen (-20ºC), except for a small working supply, which may be refrigerated for at most one week. Some labile agents (e.g. imipenem, cefaclor, and clavulanic acid combinations) may retain greater stability if stored frozen until the day of use.
- Quality control of the above procedure is to be done by using the ATCC control strains *Escherichia coli* ATCC 25922 side by side.

Figure 5: (a) AST *E.coli* ATCC 25922 (b) AST *E.coli* isolate

Reporting of results

a) For "No growth" cultures, indicate the length of incubation: "No growth after x number of days of incubation" for both preliminary and, final reports. (automated systems: 5 days, manual systems: 5-7 days).

b) Positive cultures:

i. Immediately report Gram stain results of all positive cultures to the physician in-charge, with as much interpretive information as possible.

ii. Follow immediately with a written or computer-generated final report including the following:

- Antibiotic Susceptibility Test result
- Date and time of collection and receipt
- Date and time of positive result is reported

Interpretation

a) The report of a positive culture generally means that the patient is bacteremic. However, skin microbiota may contaminate the culture, causing a false-positive result, or pseudo-bacteremia; the latter has many other causes too.

Some examples of common commensals: Coagulase Negative *Staphylococcus*, *Bacillus* species, *Corynebacterium* species (Diphtheroids), *Micrococcus*, *Propionibacterium* species

Examples of organisms recognized as pathogens: *Staphylococcus aureus, Streptococcus pyogenes, Streptococcus pneumoniae, Enterococcus faecalis, Enterococcus faecium, E. coli, Klebsiella pneumoniae, Salmonella, Pseudomonas aeruginosa, Acinetobacter species, Haemophilus influenzae, Neisseria meningitidis, Candida species*.

b) Mixed cultures can be present and account for a small but significant number of bacteremia.

All bacterial isolates are routinely tested by Kirby-Bauer disk-diffusion assay for their sensitivity to a variety of antibiotics that are currently being used for treating eye infections. The procedure adopted is based on CLSI guidelines.

Table 3: Commonly tested antibiotics against gram negative and gram positive isolates.

Gram negative (1st line)	**Gram positive**
Chloramphenicol (Himedia, Catalog:SD006-5CT)	Cefazolin (Himedia, Catalog:SD047-5CT)
Ciprofloxacin (Himedia, Catalog:SD060-5CT)	Chloramphenicol (Himedia, Catalog:SD006-5CT)
Gentamicin (Himedia, Catalog:SD016-5CT)	Ciprofloxacin (Himedia, Catalog:SD060-5CT)
Ofloxacin (Himedia, Catalog:SD087-5CT)	Cefuroxime (Himedia, Catalog:SD0615X5ODS)
Ceftazidime (Himedia, Catalog:SD062-5CT)	Ofloxacin (Himedia, Catalog:SD087-5CT)
Amikacin (Himedia, Catalog:SD035-5CT)	Vancomycin (Himedia, Catalog:SD045-5CT)

Gatifloxacin (Himedia, Catalog:SD737-5CT)	Gatifloxacin (Himedia, Catalog:SD737-5CT)
Moxifloxacin (Himedia, Catalog:SD217-5CT)	Amikacin (Himedia, Catalog:SD035-5CT)
Cefuroxime (Himedia, Catalog:SD061-5X5ODS)	Moxifloxacin (Himedia, Catalog:SD217-5CT)
Gram negative (2nd line)*	Co-trimoxazole (*Nocardia*) (Himedia, Catalog:SD010-5CT)
Piperacillin/Tazobactam (Himedia, Catalog:SD210-5CT)	Oxacillin (*Staphylococcus*) (Himedia, Catalog:SD043-5CT)
Cefoxitin (*Staphylococcus*) (Himedia, Catalog:SD041-5CT)	
Linezolid (*Staphylococcus*) (Himedia, Catalog:SD215-5CT)	
Azithromycin (*Staphylococcus*) (Himedia, Catalog:SD204-5CT)	
Bacitracin (*Streptococcus*) (Himedia, Catalog:EM126-10ST)	
Optochin (*Streptococcus*) (Himedia, Catalog:DD009- 5CT)	

1.2.2. Identification And Antibiotic Sensitivity Using Vitek 2 Compact

VITEK 2 Compact Instrument (V2C) is an automated identification system for identification and sensitivity testing of microorganisms. It utilizes growth-based technology which accommodates the colorimetric reagent cards for organism identification and colometry based antimicrobial susceptibility using MIC test cards that are incubated and interpreted automatically.

Primary sample

A pure colony of bacterial isolate (Eg. *E. coli)* that has been incubated overnight in a nutrient rich medium (e.g., nutrient agar- Himedia, Catalog: GRM666-500G) is to be used.

Equipment required

- VITEK 2 ® Compact

- VITEK 2 ® Compact Densicheck Plus (bioMérieux Inc.)

Other Consumables required

- Gram negative ID cards (bioMérieux Inc., Catalog:GN 21341)
- Gram negative AST cards (bioMérieux Inc., Catalog:AST-N235)
- Polystyrene test tube
- Cassette
- Sterile saline (aqueous 0.45% to 0.50% NaCl, pH 4.5 to 7.0) (bioMérieux Inc., Catalog: V1204)
- Inoculation loop
- Bunsen Burner
- 200-1000 µL micropipette tips (Tarsons, Catalog: 521020**)**
- 100-1000 µL micropipette (Tarsons, Catalog: 3123000063)
- Discarding Jar

Procedure

A. Suspension preparation:

Using a sterile inoculation straight wire, transfer a sufficient number of colonies of a pure culture and suspend the microorganism in 3.0 mL of sterile saline (aqueous 0.45% to 0.50% NaCl, pH 4.5 to 7.0) in a 12 x 75 mm clear plastic (polystyrene) test tube. Adjust turbidity accordingly and measure using a turbidity meter called the DensiChekTM to the range 0.50- 0.63 (specific for gram negative organism)

For identification of *E. coli,* only one test tube containing the bacterial suspension with turbidity ranging between 0.50- 0.63 is to be used.

For AST of *E. coli,* 145 µL is taken out from the bacterial suspension with turbidity ranging between 0.50 - 0.63 to another test tube containing 3 mL of sterile saline (aqueous 0.45% to 0.50% NaCl, pH 4.5 to 7.0) and mix well.

Figure 6: Suspension preparation

B. Inoculation:

A test tube containing the microorganism suspension is to be placed into the tube holder present in the special rack (cassette) and the reagent card has to be placed in the neighboring slot while inserting the transfer tube into the corresponding suspension tube. For identification of *E. coli*, the gram-negative ID cards are to be used, whereas for AST, the N235 card is to be used. The cassette can accommodate up to 10 tests. The filled cassette is to be placed manually into the filling chamber of the Vitek 2 machine. The loading button will be switched on until the whole process is over. The door of the filling chamber will be opened automatically once the process is done

Figure 7: Inoculation of isolate into reagent car

C. Card sealing and incubation:

After loading the cassette in the loader chamber, the cards will need to be sealed and loaded into the carousel incubator. Data are to be collected at 15-minute intervals during the entire incubation period. The VITEK system analyses the data results and determines the identification and sensitivity of the test organism. Result data is automatically recorded and generated by the computer in the form of a printout. The printout for the identification and sensitivity of the test organism will be filed in the VITEK 2 Compact Software.

D. **Standardization of DensiCHEKTM Plus using DensiCHEKTM Plus standard kit:** DensiCHEKTM plus standards are used to verify the DensiCHEKTM plus instrument measurement performance. Use of three standards enables the accuracy of the instrument to be monitored and therefore confirm accuracy of the organism suspension.

Figure 8: Standardization of DensiCHEKTM Plus

Precautions need to be taken when using the tubes. Do not open the tubes and do not shake any of the standard tubes, since air bubbles can affect readings. It is recommended to use the standard kit to test accuracy of the standard tube into the DensiCHEKTM Plus prior to first use and on a monthly basis.

Important instructions for use:

- Confirm that the DensiCHEKTM Plus instrument is set to the GLASS tube setting.
- Select the 0.0 McF standards and clean the outside surface with a lens tissue.
- Ensure the instrument is on and insert the 0.0 McF standards into the instrument and press the Zero/Scroll key.
- Slowly rotate the standard one full rotation. The instrument will display a series of dashes followed by 0.00.
- Select the desired standard (0.5, 2.0, and 3.0) and clean outside surface with lens tissue.
- Insert the standard into the instrument and slowly rotate the standard one full rotation until a numerical value is displayed.

- Check that the displayed McFarland value is within the acceptable range.
- If any standards are outside the acceptable range, repeat above mentioned steps. If it is still out of the range contact with vendors.

Table 4: Reference range

Standard	**Acceptable Range**
0.00 McF	0.00
0.5 McF	0.44 - 0.56
2.0 McF	1.85 – 2.15
3.0 McF	2.79 – 3.21

E. Quality control:

The following organisms are used for quality controls:

ATCC *Enterococcus faecalis* (25912)	ATCC *Staphylococcus aureus* (25923)	ATCC *Escherichia coli* (25922)	ATCC *Klebsiella pneumonia* (700603)

Frequency of testing will be once every week using all the cards in use.

1.2.3. Identification of pathogen of interest

Example: Identification of *E.coli*

After overnight incubation, if there is growth, identification is to be done based on the colony characteristics. Gram staining is required to verify the growth of gram-negative organism.

The following biochemical tests are to be performed so as to confirm that the organism is *E. coli*:

Gram stain (gram negative bacilli), Catalase production (negative), Oxidase production (negative), Methyl red test (positive), Voges-Proskaur test (negative), Indole ring test (positive), Triple Sugar Iron test (A/A), Citrate utilisation test (negative), Urease production test (negative) and motility test (motile).

Final verification is done using the automated Vitek 2 system (refer to the chapter "Identification and AST Using the Vitek 2 Compact system", pages 17-20).

1.3. Standard operating procedure for DNA Extraction

DNA extraction of gram positive and gram negative bacteria from clinical samples can be carried out using various commercially available kits such as Qiagen and Wizard Promega.

1.3.1. DNA Extraction for gram positive isolates

Requirements

- Qiagen DNeasy kit (Qiagen, Catalog:69504/69506)
- Water bath or thermomixer at 56℃ & 70℃ (or 56℃ & 37℃ for gram positive isolates)
- Pre-warmed 10mM Tris-Cl (to be used as Elution buffer) (Himedia, Catalog:DS0040)
- Ethanol (Analytical CSS Reagent, SKU: UN1170)
- Lysozyme 10mg/ml (for gram positive isolates) (Himedia, Catalog:MB098-5G)

Protocol for DNA extraction is the same for Gram-positive and Gram-negative organisms except for the cell-lysis steps. Please see the details below.

- Pick identical looking colonies from freshly plated culture and prepare 2 ml suspension in WFI. (Such that culture density corresponding to 4 McFarland i.e., $1.2*10^9$ cfu/ml is obtained). This cell suspension can be further used for DNA extraction.
- Transfer this suspension to Eppendorf (microcentrifuge tubes) and centrifuge for 10 mins at 7500rpm.
- Discard the supernatant using micropipette.
- Resuspend pellet in 50µl lysozyme and incubate for 1 hour @ 37℃*
- Add 25µl Proteinase K and 200µl Buffer AL (without ethanol). Mix by vortexing.
- Incubate at 56°C for 30 min.

  NOTE: For the above steps, the protocol varies based on gram nature of organism. Use the appropriate lysis steps as indicated.
- Add 200µl of absolute ethanol (96-100%) and mix by pulse vortexing for 15 seconds.

  NOTE: It is important that the sample and the ethanol are mixed thoroughly to yield a homogeneous solution. A white precipitate may form on addition of ethanol.

It is essential to apply all of the precipitate to the DNeasy Mini spin column. This precipitate does not interfere with the DNeasy procedure.

- Pipette the entire mixture from above step (including any precipitate) into the DNeasy Mini spin column placed in a 2 ml collection tube (provided with kit). Centrifuge at 8000 rpm for 1 min.
- Discard flow-through and collection tube. (if there are not enough tubes, you can discard the liquid inside the collection tube and re-use it).
- Place the DNeasy Mini spin column in a new 2 ml collection tube (provided with kit), add 500µl Buffer AW1, and centrifuge for 1 min at 8000 rpm. Discard flow-through and collection tube. (if there are not enough tubes, you can discard the liquid inside the collection tube and re-use it).
- Place the DNeasy Mini spin column in a new 2 ml collection tube (provided with kit), add 500µl Buffer AW2 and centrifuge for 3 min at 14,000 rpm to dry the DNeasy membrane.
- Discard flowthrough and collection tube and repeat the step by centrifuging at 14000 rpm for 1 min.

  NOTE: It is important to dry the membrane of the DNeasy

Note: Qiagen kit can also be used for DNA extraction of gram-negative organisms from clinical samples.

1.3.2 DNA Extraction from gram negative Isolates

The extraction of *E. coli*/gram negative AMR isolates can be done using commercially available kits such as the Wizard® HMW DNA Extraction Kit A2920 from Promega is described below:

Product Components

- 50ml HMW Blood Lysis Buffer (Promega, Catalog: A293A)
- 25ml HMW Lysis Buffer A (Promega, Catalog: A294A)
- 1.0ml Proteinase K Solution (Promega, Catalog:MC500C)
- 25ml Protein Precipitation Solution (Promega, Catalog: A795A)
- 50ml DNA Rehydration Solution (Promega, Catalog: A796A)
- 250µl RNase A Solution (Promega, Catalog: A797A)

Storage Conditions

Store the Wizard® HMW DNA Extraction Kit at room temperature (+15°C to +30°C). See product label for expiration date.

Procedure:

Purifying genomic DNA from bacteria works best when cultures are grown for 14 hours or less, ideally in exponential growth phase. Cultures grown for longer tend to produce large amounts of protein and RNA that can affect the performance and purity of the isolated genomic DNA.

Materials required

- Sterile 1.5ml microcentrifuge tubes (Tarsons, Catalog:500010)
- Phosphate-buffered saline (PBS) (Sigma-Aldrich, Catalog: P4417-100TAB)
- 80°C water bath
- 37°C water bath
- 56°C water bath
- Isopropanol, room temperature (ThermoFisher Scientific, Catalog:Q26897)
- 70% ethanol, room temperature
- Wide-bore pipette tips (1000 µl (Tarsons, Catalog:521020) and 200 µl (Tarsons, Catalog:521010))
- Optional: 65°C water bath (for rapid DNA rehydration)

1. Add 1ml of an overnight culture to a 1.5 ml microcentrifuge tube.
2. Centrifuge at 13,000–16,000 × g for 2 minutes to pellet the cells. Remove the supernatant. For Gram-negative bacteria, resuspend the cells thoroughly in 100 µl of PBS.
3. Add 500 µl of HMW Lysis Buffer A. Using 1000 µl wide bore pipette tips, mix the solution five times to lyse the cells. Draw the tube contents slowly from the bottom of the tube, then expel the lysate rapidly down the side of the tube. The solution will become very viscous. Do not pipette more than five times to avoid DNA shearing. *Note: If wide-bore pipette tips are unavailable, use standard 1000 µl pipette tips for mixing sample with HMW Lysis Buffer A.*
4. If lysis appears incomplete, incubate at 80°C for 5 minutes to lyse the cells then cool to room temperature.

5. Add 3 µl of RNase A Solution to the cell lysate, and mix by inverting the tube 5–7 times. Incubate the mixture at 37°C for 15 minutes.

6. Add 20 µl of Proteinase K Solution to each sample and mix by inverting the tube 10 times. Incubate the mixture at 56°C for 15 minutes. Cool to room temperature for at least 5 minutes or chill on ice for 1 minute.

7. Add 200 µl of Protein Precipitation Solution to the cell lysate. Using 1000 µl wide bore pipette tips, mix the solution five times. Draw the tube contents from the bottom of the tube, then expel the lysate rapidly down the side of the tube. Small protein clumps may be visible after mixing. Incubate on ice for 5 minutes. Note: If wide-bore pipette tips are unavailable, vortex lysate and Protein Precipitation Solution for 5 seconds. Do not tip mix.

8. Centrifuge at 13,000–16,000 × g for 10 minutes at room temperature. A protein pellet will be visible. If any unpelleted debris is visible, repeat the centrifugation step.

9. Slowly transfer the supernatant to a clean 1.5 ml microcentrifuge tube by decanting the sample into a tube containing 600 µl of room-temperature isopropanol.

Note: Some supernatant may remain in the original tube containing the protein pellet. Leave this residual liquid in the tube to avoid contaminating the DNA solution with the precipitated protein.

10. Gently mix the solution by gently inverting the tube eight times. Incubate for 1 minute at room temperature and repeat the inversion. White thread-like strands of DNA may form a visible mass.

11. Centrifuge at 13,000–16,000 × g for 2 minutes at room temperature. The DNA will be visible as a small white pellet.

12. Decant the supernatant and add 600µl of room temperature 70% ethanol to the DNA. Gently invert the tube several times to wash the DNA pellet and the sides of the microcentrifuge tube. Centrifuge as instructed in Step 11.

13. Carefully aspirate the ethanol. The DNA pellet is very loose at this point so carefully avoid disturbing or aspirating the pellet into the pipette. Invert the tube on clean absorbent paper and air-dry the pellet for 10–15 minutes.

14. Add 100 µl of DNA Rehydration Solution to the tube. Do not vortex because this will cause mechanical shearing and decrease average fragment size. Rehydrate the DNA by incubating the solution overnight at room temperature. Alternatively, incubate the purified DNA at 65°C for 1 hour, periodically mixing the solution by gently tapping the tube.

15. If DNA appears non-homogeneous (e.g., undissolved pellet is still visible), mix with 200 µl wide-bore pipette tips. Store the DNA at 2–8°C.

Note: The Wizard Promega kit can also be used for DNA extraction of gram-negative organisms from clinical samples.

1.3.3 Check DNA Quality

Ensure that required quality standards are met. The concentration requirements might vary depending on the sequencing platform, however, as a general rule of thumb the following quality and purity should be maintained:

Quality conditions required for Illumina sequencing:

Final concentration required ~50 ng/µl

Total 2-4 µg of RNA-free genomic DNA (NGS grade) of molecular weight >40kb.

Nanodrop quality check - A260/280 ratio >1.8; A260/230 = 2.0- 2.2.

Quality conditions required for Nanopore sequencing for high quality assembly

Final DNA concentration of ~100 ng/µl

Nanodrop quality check - A260/280 ratio >1.8; A260/230 = 2.0- 2.2

DNA intactness as detected by agarose gel electrophoresis or tapestation DIN values of minimum 4.

Label the extracted DNA properly and store at -20˚C until the time for sequencing.

2 Library Preparation by Native Barcoding Kit 96 V14 (SQKNBD114.96) For Oxford Nanopore Platform

Reagent/solution requirement:

- Nuclease-free water
- Qubit 1X dsDNA HS solution
- NEBNext FFPE DNA Repair Buffer
- Ultra II End-prep Reaction Buffer
- Ultra II End-prep Enzyme Mix
- NEBNext FFPE DNA Repair Mix
- Native Barcode (NB01-96)
- Blunt/TA Ligase Master Mix
- EDTA (EDTA)

- AMPure XP Beads (AXP)
- 80% ethanol
- Native Adapter (NA)
- NEBNext Quick Ligation Reaction Buffer (5X)
- Quick T4 DNA Ligase
- Short Fragment Buffer (SFB)
- Elution Buffer (EB)
- Flow Cell Tether (FCT)
- Flow Cell Flush (FCF)
- Sequencing Buffer (SB)
- Library Beads (LIB)

2.3 Quality Check (Concentration measurement by Qubit fluorometer)

a) Add 01 µl + 199 µl Qubit 1X dsDNA HS solution in Qubit tube
b) Mix by vortexing and give short spin
c) Incubate for at least 2 min at room temperature
d) Measure the concentration by using Qubit fluorometer

2.4 Library preparation and sequencing

a) DNA repair and end-prep:

i) In a clean 96-well plate, aliquot 400 ng DNA per sample.
ii) Make up each sample to 12 µl using nuclease-free water. Mix gently by pipetting and spin down.
iii) Add the following components per well:

Reagent	**Volume**
DNA sample	11 µl
NEBNext FFPE DNA Repair Buffer	1 µl
Ultra II End-prep Reaction Buffer	0.875 µl
Ultra II End-prep Enzyme Mix	0.875 µl
NEBNext FFPE DNA Repair Mix	0.75 µl
Total	**0.5 µl**

iv) After addition, pipette mix 10-20 times.
v) Ensure the components are thoroughly mixed by pipetting and spin down briefly.
vi) Using a thermal cycler, incubate at 20°C for 5 minutes and 65°C for 5 minutes.

b) Native barcode ligation:

i) Add the following components per well:

Reagent	Volume
End-prepped DNA	3.75
Native Barcode (NB01-96)	0.75 µ
Blunt/TA Ligase Master Mix	1.25 µl
Total	**10 l**

ii) After addition, using pipette mix 10-20 times.

iii) Thoroughly mix the reaction by gently pipetting and briefly spinning down.

iv) Add 1 µl of EDTA to each well and mix thoroughly by pipetting and spin down briefly.

v) Resuspend the AMPure XP Beads (AXP) by vortexing.

vi) Add 4 µl AMPure XP Beads (AXP) to each well, and mix by pipetting for a 0.4X clean.

vii) Incubate on a Hula mixer (rotator mixer) for 10 minutes at room temperature.

viii) Prepare fresh 80% ethanol in nuclease-free water.

ix) Spin down the sample and pellet on a magnet for 5 minutes. Keep the plate on the magnetic rack until the eluate is clear and colourless, and pipette off the supernatant.

x) Keep the plate on the magnetic rack and wash the beads with 80 µl of freshly prepared 80% ethanol without disturbing the pellet. Remove the ethanol using a pipette and discard.

xi) Repeat the previous step.

xii) Spin down and place the tube back on the magnetic rack. Pipette off any residual ethanol. Allow the pellet to dry for ~30 seconds, but do not dry the pellet to the point of cracking.

xiii) Remove the plate from the magnetic rack and resuspend the pellet in 12 µl nuclease-free water by pipette mixing.

xiv) Incubate for 10 minutes at 37°C, after, give a short spin.

xv) Pellet the beads on a magnetic rack until the eluate is clear and colourless.

xvi) Remove and retain 11 µl of eluate into a clean new 96 well plate.

xvii) Quantify 1 µl of eluted sample using a Qubit fluorometer

c) Adapter ligation and clean-up:

i) Aliquot and pool 50 ng of each Native barcoded sample in a 1.5 ml Eppendorf LoBind tube.

ii) Measure the volume of pooled Native barcoded sample, if it is more than 30 µl then do speedvac Concentrator to reduce volume up to 30 µl.

iii) Add the following components in order in a 1.5 ml tube containing pooled Native barcoded samples.

Reagent	**Volume**
Pooled barcoded sample	30 µl
Native Adapter (NA)	5 µl
NEBNext Quick Ligation Reaction Buffer (5X)	10 µl
Quick T4 DNA Ligase	5 µl
Total	**50 µl**

iv) Thoroughly mix the reaction by gently pipetting and briefly spinning down.

v) Incubate the reaction for 20 minutes at room temperature.

vi) Resuspend the AMPure XP Beads (AXP) by vortexing.

vii) Add 20 µl of resuspended AMPure XP Beads (AXP) to the reaction and mix by pipetting.

viii) Incubate on a Hula mixer (rotator mixer) for 10 minutes at room temperature.

ix) Spin down the sample and pellet on the magnetic rack. Keep the tube on the magnet and pipette off the supernatant.

x) Wash the beads by adding 125 µl Short Fragment Buffer (SFB). Flick the beads to resuspend, spin down, then return the tube to the magnetic rack and allow the beads to pellet. Remove the supernatant using a pipette and discard.

xi) Repeat the previous step.

xii) Spin down and place the tube back on the magnet. Pipette off any residual supernatant. Allow to dry for ~30 seconds, but do not dry the pellet to the point of cracking.

xiii) Remove the tube from the magnetic rack and resuspend the pellet in 25 µl Elution Buffer (EB).

xiv) Spin down and incubate for 10 minutes at 37°C. Every 2 minutes, agitate the sample by gently flicking for 10 seconds to encourage DNA elution.

xv) Pellet the beads on a magnet until the eluate is clear and colourless, for at least 1 minute.

xvi) Remove and retain 25 µl of eluate containing the DNA library into a clean 1.5 ml Eppendorf DNA LoBind tube.

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

2.5 Priming and loading the PromethION flow cell

i) Prepare the flow cell priming mix in a suitable tube for the number of flow cells to flush. Once combined, mix well by briefly vortexing.

Reagent	**Volume per flow cell**
Flow Cell Tether (FCT)	30 µl
Flow Cell Flush (FCF)	1170 µl
Total volume	**1,200 µl**

ii) Load the flow cell(s) into the docking ports of the PromethION instrument.

iii) Open the inlet port by turning the valve clockwise.

iv) After opening the inlet port, draw back a small volume to remove any air bubbles:

- Insert the tip into the inlet port.
- Turn the wheel until the dial shows 220-230 µl, or until you see a small volume of buffer entering the pipette tip.
- Load 500 µl of the priming mix into the flow cell via the inlet port, avoiding the introduction of air bubbles. Wait five minutes.

v) Thoroughly mix the contents of the Library Beads (LIB) by pipetting.

vi) Calculate the volume required for 20 fmol and make the final volume 32 µl by adding an elution buffer.

vii) In a new 1.5 ml Eppendorf DNA LoBind tube, prepare the library for loading as follows:

Reagent	**Volume per flow cell**
Sequencing Buffer (SB)	100 µl
Library Beads (LIB)	68 µ
DNA library	32 µl
Total	**200 µl**

viii) Complete the flow cell priming by slowly loading 500 µl of the priming mix into the inlet port.

ix) Mix the prepared library gently by pipetting up and down just prior to loading.

x) Using a Pipette 1000, insert the pipette tip into the inlet port and load 200 µl of the library.

xi) Close the valve to seal the inlet port.

3 Computational pipeline for whole genome assembly and ARGs identification

3.3 QC and Sequence assembly

Quality Control (QC) and assembly of sequencing results are crucial steps in the analysis of genomic data. The integrity and accuracy of the assembled genome directly impact the conclusions drawn from the data. This section outlines the process for QC and assembly of genomes sequenced using Illumina and ONT platforms, with an emphasis on tools and pipelines available for bioinformaticians familiar with Linux/Bash scripting and bioinformatics analysis.

Figure 9.1) Short-read

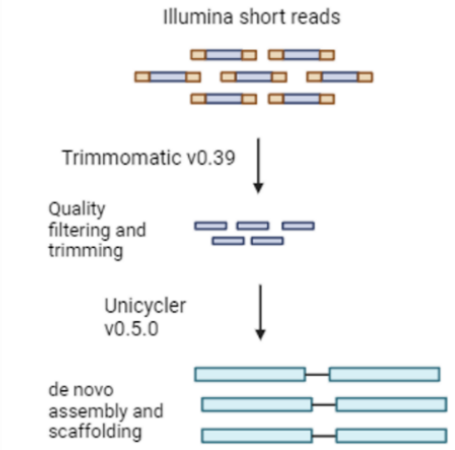


Figure 9.3) Hybrid Assembly

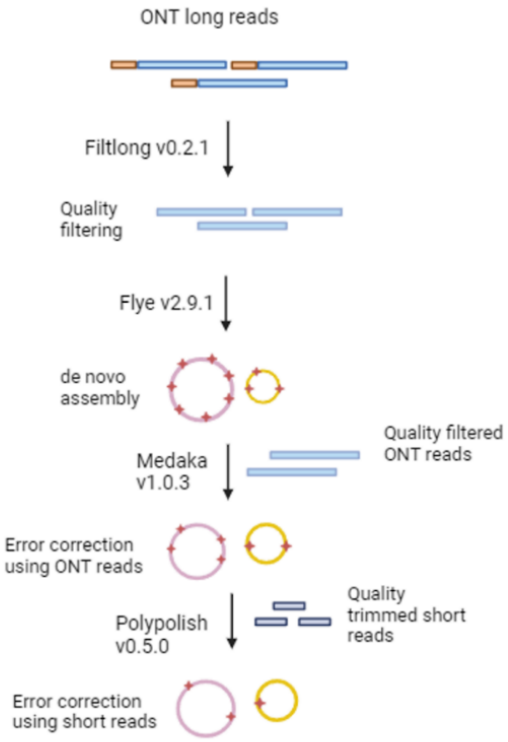


Figure 9.2) Long-read

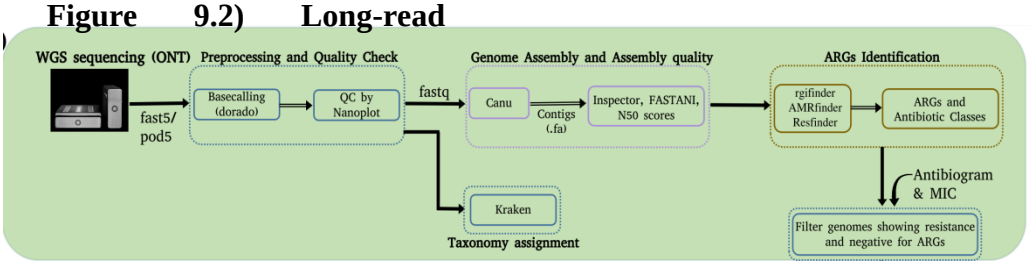


Figure 9: Computational pipeline for genome assembly using three different approaches.

Figure 9.1 shows the assembly workflow using the data from Illumina platform alone. Figure 9.2 shows genome assembly and AMR analysis pipeline for long reads data using ONT platform. Figure 9.3 shows a hybrid approached where the same genome is sequenced on the ONT as well as Illumina platforms to achieve high-quality genomic assemblies. Please note that similar, alternative tools can be used as well.

3.3.2 Quality Control (QC)

For Illumina Data:

FastQC: A widely used tool for quality checking of Illumina sequence data. It provides a quick overview of the data quality, including base quality score distributions, GC content, and sequence duplication levels.

For ONT Data:

NanoQC: Offers quality control metrics specifically for long-read sequencing data, such as read length distribution and quality scores.

3.3.3 Bacterial Genome Assembly methods

Different sequencing platforms require specific pipelines for optimal genome assembly. Here, we present three distinct approaches to construct assemblies based on the sequencing platform used.

Illumina (Short-read) assembly:

A common approach for short-read assembly is using tools like Unicycler or MaSuRCA (complex genomes). These tools are designed to handle high-throughput short-read sequences and can efficiently assemble bacterial genomes (Figure 9.1). The pipeline for assembling short-read data is available at (https://github.com/Vasundhara-Karthik/IlluminaBacSeq.git)

Long-read Assembly:

Pod5/fast5 files from ONT are basecalled using the 'Super Accuracy model' of Dorado (basecaller). Quality check on raw reads is done using nanostat. Fastq files are further assembled into fasta files with Canu (Genome assembler) (Koren *et al.*, 2017). Inspector (Quality check tool) is used to check the assembly quality (Figure 9.2)

Additionally, EPI2ME platform is a cloud-based data analysis service developed by Metrichor Ltd., a subsidiary of ONT. The EPI2ME platform offers a range of analysis workflows, e.g. for metagenomic identification, barcoding, alignment, and structural variant calling. The analysis requires no additional equipment or compute power, and provides an easy-to-interpret report with the results.

ONT (Long-read) first hybrid assembly:

For long-read or hybrid assemblies (combining Illumina and ONT data), tools like Flye (for long reads), Canu and Unicycler (for short-read first hybrid assembly) are recommended. These tools are capable of handling the larger, more complex reads from the ONT platform (Figure 9.3).

ONT hybri-flow pipeline for long-read first hybrid assembly, optimizing the use of both long and short-read data for comprehensive genome assembly is provided at (https://github.com/Vasundhara-Karthik/ONThybri-flow.git). After assembly, validating the quality of the assembled genome is essential. Tools like QUAST and BUSCO can evaluate the contiguity, completeness and correctness of the assembly.

3.4 ARGs annotation

To identify ARGs in assembled genomes, RGIfinder can be used with CARD (Comprehensive Antibiotic Resistance Database) (Alcock *et al*. 2023) as reference database. Additionally, other tools like AMRfinderPlus, Resfinder can be used.

3.5 Mobile genetic elements (MGEs) Annotation

Plasmidfinder (Carattoli *et al*., 2014) and Genomad (Camargo *et al*., 2023) can be used to annotate contigs as plasmids from the assembled genomes. Further, ISfinder (https://isfinder.biotoul.fr/) will help us to annotate other MGEs in the identified

plasmid sequences. This is important for understanding the mechanisms of horizontal gene transfer.

3.6 Antibiogram and ARGs Integration

In house python or R or perl scripts can be written to consolidate the phenotypic outcomes from antibiogram with ARGs identified from the genomes.

3.7 Data Visualization

Phyloseq (McMurdie *et al*., 2013), Microbiome (Lahit and Shetty 2012), ggplot and ggpubr packages in R are used for downstream analysis and data visualization.

The resulting data and analysis can significantly aid physicians and researchers in understanding the distribution and dynamics of AMR genes within a population or a clinical setting. This knowledge is crucial for managing and controlling the spread of antimicrobial resistance.

4 Outcomes

4.3 Bacterial Genome Assembly

With this protocol, near finished and high fidelity bacterial genomes can be constructed from pure culture isolates using long read sequencing technology. Figure 10 shows high quality genomes of Pseudomonas aeruginosa and Staphylococcus aureus constructed using long read assembly pipeline.

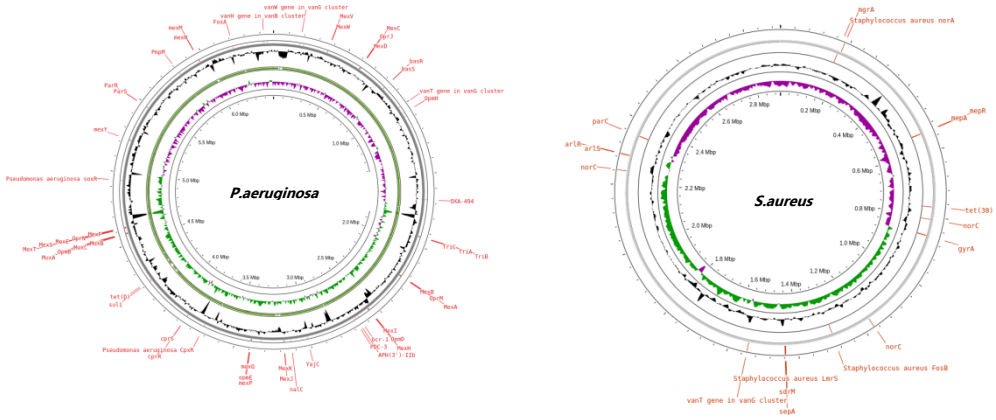


Figure 10. Near finished genome assemblies of *P.aeruginosa* and *S.aureus* with ANI >98% using ONT. Assemblies have one contig representing whole bacterial genome with high N50 scores.

4.4 Antibiogram

An antibiogram is typically a resistance-sensitivity pattern based on the phenotypic tests. A plot describing the resistance profiles of a group of isolates, can help a physician understand the dominant resistance profiles in circulation and thus establish appropriate treatment plans. Antibiogram for several isolates ranging over a period of time can indicate prevalence as well as the evolution of resistance. Such output can be used in surveillance studies or to track an ongoing outbreak. Figure 11 is an example of the analysis from antibiograms (which can be derived either phenotypically or genotypically) and is especially useful for understanding prevalence and evolution of resistance

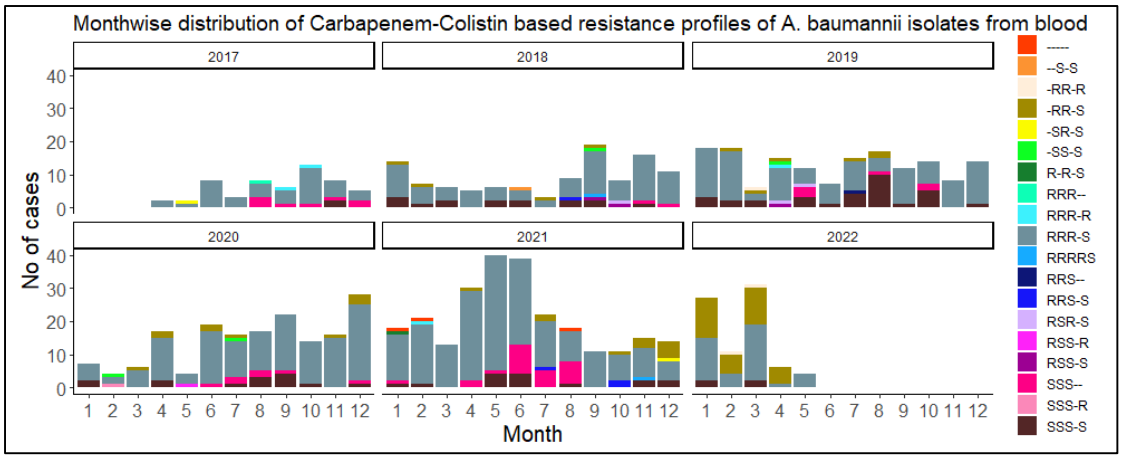


Figure 11. Distribution of carbapenem – colistin resistance profiles of multiple A. baumannii isolates over a period of 5 years.

4.5 Phenotype and genotype integration

a) Detailed analysis of antibiogram can summarize the proportion of pathogenic organism that are resistance or susceptible to different antibiotics.

   i. Figure 12 below summarizes percentage of samples resistant or susceptible to antibiotics across different isolates.

ii. *K. pneumoniae* and *E. hormaechei* showed high resistance rates to all classes of antibiotics. *S.aureus* isolates showed high resistivity to quinolones but sensitive to carbapenems (imipenem, meropenem) and glycopeptide antibiotics.

b) ARGs identified from genomes of each isolate is integrated with antibiogram results to understand the concurrence of ARGs prevalence and antibiotic reactivity. Figure 13 shows consolidated results from antibiogram and ARGs distribution across 55 isolates.

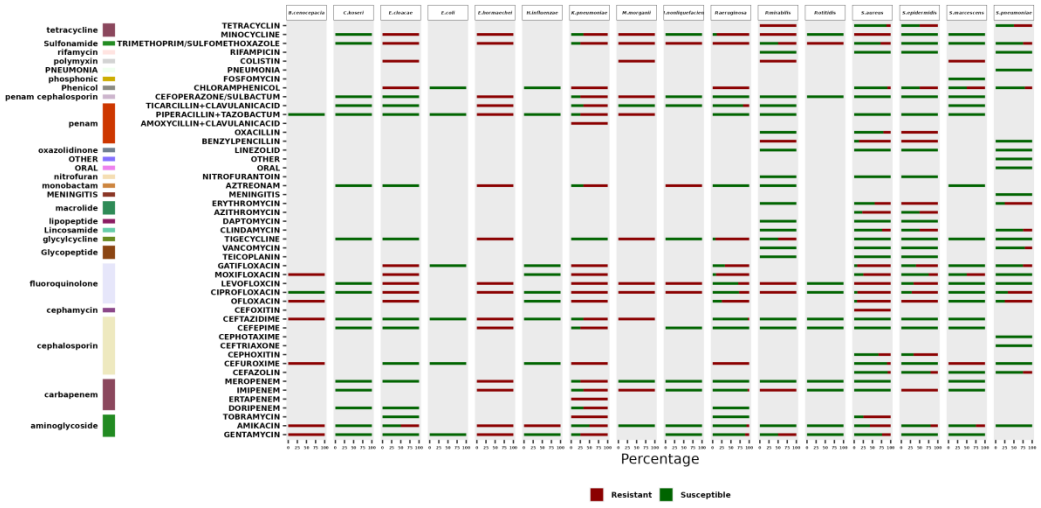


Figure 12. Resistant and susceptibility rates of bacterial isolates to different classes of antibiotics

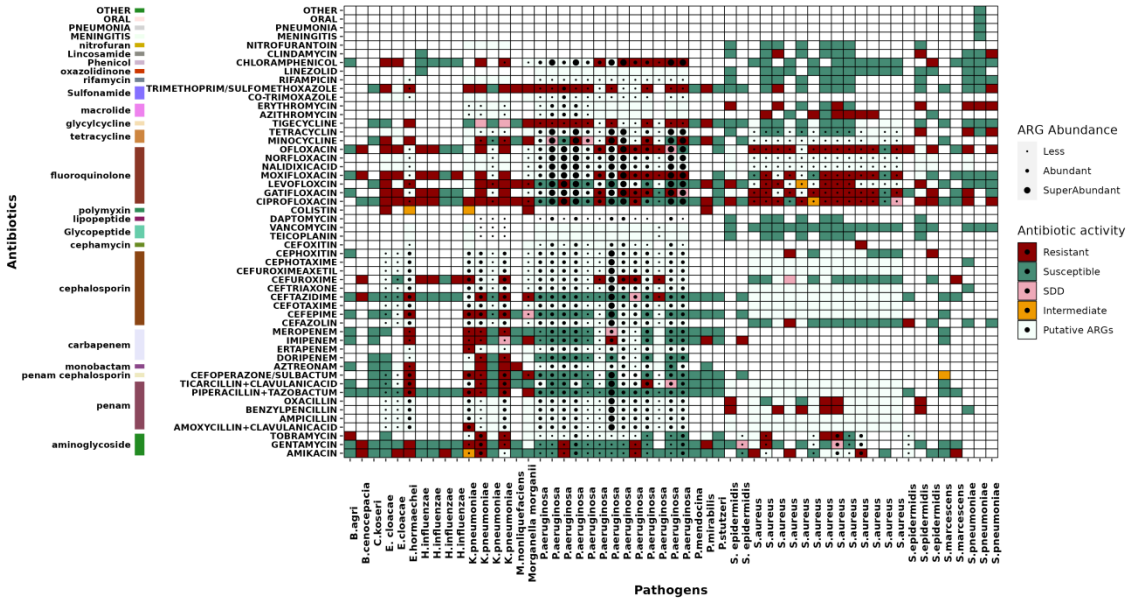


Figure 13. Consolidated results from antibiogram and ARGs prevalence

4.6 Prevalence of ARGs in pathogens

These charts depict the prevalence of ARGs in each pathogenic species or group. Some of these ARGs can be correlated with specific drug resistance patterns. Thus prevalence chart can be used to establish therapeutic plans based on AMR gene prevalence and surveillance. Figure 14 shows the distribution of important carbapenem resistance genes in *Klebsiella* spp. isolates.

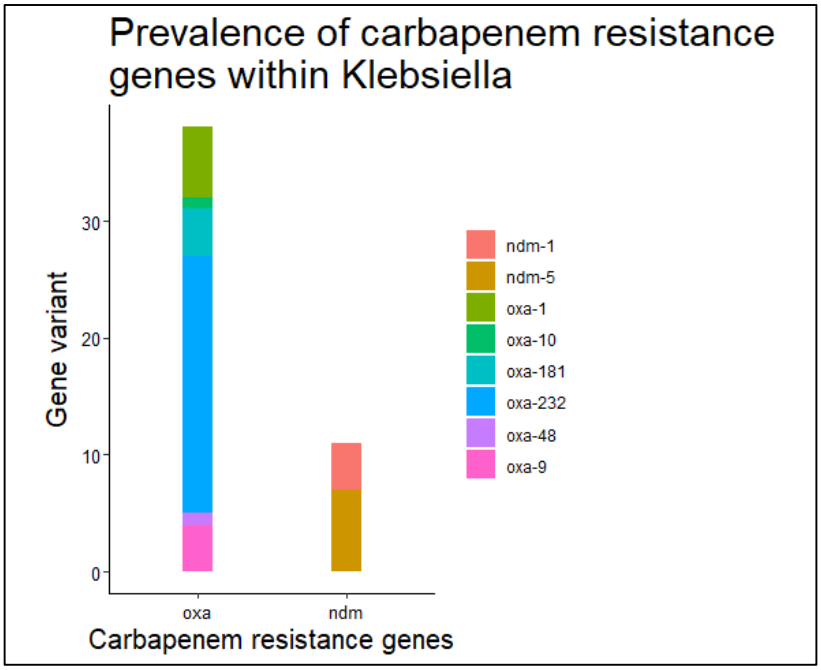


Figure 14. Distribution of ARGs conferring resistance to carbapenem in the *Klebsiella* spp. isolates.

4.7 Horizontal gene transfer of ARGs in bacterial isolates

a) Identification of mobile genetic elements including plasmids, insertion elements, transposons helps to understand the dissemination of ARGs from one bacteria to other through conjugation

b) Genetic communication between bacterial species in disseminating ARGs can be derived from presence of ARGs in plasmids and their relationships among isolates. Figure 15 shows ARGs carried by plasmids of *E. cloacae* and *K. pneumoniae*. This study helps to identify sequence similarity of ARGs

carrying plasmids to further understand horizontal gene transfer of ARGs and possible genetic communication across species.

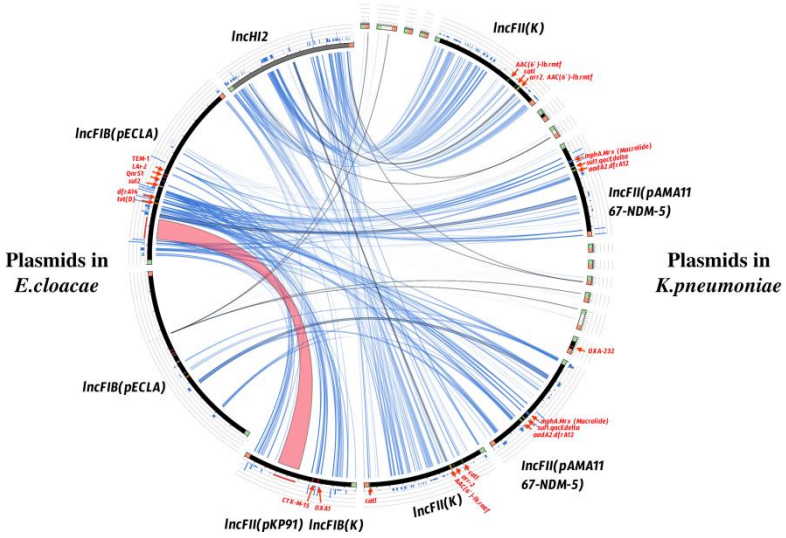


Figure 15. Plasmids relationship in *E. cloacae* and *K. pneumoniae*

Prevalence and dissemination of ARGs is crucial clinical implications of AMR across worldwide. This protocol and outcomes will strengthen knowledge of ARG prevalence and surveillance in health care facilities. Also, it will significantly assist physicians to understand resistance proportions of critical antibiotics along with genomic signatures and their transmission risk. This could further aid in correct selection of antibiotics for a given set of population.

5 References:

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