Cultivation and Genomic DNA Extraction of *Klebsiella pneumoniae*

Drew A. Pariseau,[1] Brooke E. Ring,[1] Saroj Khadka,[1] and Laura A. Mike[1,2]

[1]Medical Microbiology and Immunology, University of Toledo College of Medicine and Life Sciences, Toledo, Ohio
[2]Corresponding author: *Laura.Mike@UToledo.edu*

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Klebsiella pneumoniae is a Gram-negative, rod-shaped bacterium of medical significance. It typically exists as part of the normal flora of the human intestine but can cause severe infections in the healthcare setting due to its rapid acquisition of antibiotic resistance. Cultivating and extracting genomic DNA from this bacterium is crucial for downstream characterization and comparative analyses. To provide a standardized approach for growing *K. pneumoniae* in the laboratory setting, this collection of protocols provides step-by-step procedures for routine culturing, generating growth curves, storing bacteria, and extracting genomic DNA. © 2024 The Authors. Current Protocols published by Wiley Periodicals LLC.

Basic Protocol 1: Reviving *K. pneumoniae* from frozen stocks
Basic Protocol 2: Cultivating *K. pneumoniae* in rich growth medium
Alternate Protocol: Cultivating in minimal liquid growth medium
Basic Protocol 3: Enumerating *K. pneumoniae* colony forming units
Basic Protocol 4: Growth curves
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Basic Protocol 6: Characterizing *K. pneumoniae* strains based on genomic sequence
Basic Protocol 7: Storage of *K. pneumoniae* frozen stocks in glycerol
Basic Protocol 8: Storage of *K. pneumoniae* in agar stabs

Keywords: culture • genome extraction • growth • maintenance • *Klebsiella pneumoniae* • storage

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INTRODUCTION

Klebsiella pneumoniae is a member of the *Enterobacteriaceae* family of bacteria and is associated with hospital-acquired urinary tract infections, septicemia, and pneumonia (Magill et al., 2014). This species causes approximately 10% of all nosocomial infections and is frequently found to be resistant to empirical antimicrobial treatments, such as carbapenem (Magill et al., 2014). Like other members of the *Enterobacteriaceae* family, members of *Klebsiella* do not require special growth medium and will grow on common growth media such as LB and MacConkey media.

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K. pneumoniae has similar growth conditions to other *Enterobacteriaceae*. The bacterium has an optimal growth temperature of between 35°C to 37°C and a pH of 7.2 (Ristuccia & Cunha, 1984). In some instances, temperatures of 30°C may be preferable to prevent overgrowth in assays visualizing colonies, such as counting colony forming units (CFUs). *K. pneumoniae* are also facultative anaerobes and can survive solely on citrate and glucose as carbon sources and ammonia as a nitrogen source (Ristuccia & Cunha, 1984).

This article describes general methods for growing colonies on LB agar, cultivating in LB-based and M9-based liquid media, generating growth curves, extracting genomic DNA extraction, performing genomic analyses, and long-term storage. Using the outlines

Table 1 Commonly Used *K. pneumoniae* Laboratory Strains

Strain (alternate names)	Notable information	Reference/source
342	ST146 *K. variicola* isolated from interior of nitrogen-efficient maize plants; pKP187; previously described as *K. pneumoniae*	Chelius & Triplett, 2000; Fouts et al., 2008
1084	ST23 K1, non-mucoid	Lin et al., 2012
AJ218	Nosocomial urinary tract infection isolate	Jenney et al., 2006
ATCC 43816 (KPPR1/KPPR1S/ MKP220)	ST493 clinical pneumonia isolate; O1:K2	Bakker-Woudenberg et al., 1985; Roosendaal et al., 1985
	KPPR1: Rifampin-resistant derivative; used to generate an arrayed transposon library	Lawlor et al., 2005; Broberg et al., 2014; Mike et al., 2021
	KPPR1S: Streptomycin- and rifampin-resistant derivative	Walker et al., 2019
	MKP220: Spectinomycin-resistant derivative; used to generate an arrayed transposon library	Paczosa et al., 2020
BJ1	K2; encodes no phages and few defense systems.	Blin et al., 2017
CG43	ST86 hypermucoid bacteremia isolate with K2 and pLVPK; highly virulent in mice	Peng et al., 1991
hvKP1	hvKp isolated from blood and liver abscess in Buffalo, NY, USA	Pomakova et al., 2012; Russo & Gill, 2013
KP-1	CG10193 environmental isolate	Lee et al., 2013
Kp52.145 (B5055/52145)	ST66 hvKp strain with O1:K2, Kp52.145pI and Kp52.145pII (pKP100)	Goslings et al. 1936; Lery et al., 2014
KPNIH1 (MKP103)	ST258 nosocomial outbreak strain isolated from the groin of an organ transplant patient; pAAC154-a50, pKPN-498, pKpQIL-6e6 (bla_{KPC})	Snitkin et al., 2012
	MKP103 is a derivative cured of pKpQIL-6e6 (bla_{KPC}) used to generate an arrayed transposon library	Ramage et al., 2017
LM21	Nosocomial isolate	Favre-Bonte et al., 1998
MGH78578 (ATCC 700721)	ST52 sputum isolate with K52 pKPN3, pKPN4, pKPN5, pKPN6, pKPN7	McClelland et al., 2001; Ogawa et al., 2005
SGH10	ST23 (CG23-I) hvKp isolate from human liver abscess with K1, pK2044-like and typical CG23-I virulence loci intact	Lam et al., 2018
TOP52 1721 (TOP 52)	Acute cystitis isolate	Rosen et al., 2008; Johnson et al., 2014
NTUH-K2044	ST23 hvKp isolate from a patient with liver abscesses and meningitis; O1:K1; pK2044	Wu et al., 2009

provided, the reader will be able to cultivate and purify DNA from *K. pneumoniae* for the use in studying growth kinetics, the effects of specific nutrients on growth, down-stream genetic analyses and manipulation, and other biochemical assays such as quantifying capsule production (Khadka et al., 2023a; Ring et al., 2023).

CAUTION: *K. pneumoniae* is a Biosafety Level 2 [BSL-2] pathogen. Follow all appropriate guidelines and regulations for the use and handling of pathogenic microorganisms.

STRATEGIC PLANNING

Strain Selection

K. pneumoniae has several strains commonly used in the laboratory. Prior to choosing a *K. pneumoniae* strain, identify the experimental purpose and downstream applications. A list of commonly used strains can be found in Table 1.

NOTE: All protocols involving animals must be reviewed and approved by the appropriate Animal Care and Use Committee and must follow regulations for the care and use of laboratory animals. Appropriate informed consent is necessary for obtaining and use of human study material.

REVIVING *K. pneumoniae* FROM FROZEN STOCKS

BASIC PROTOCOL 1

K. pneumoniae can be stored long-term at −80°C when diluted in sterile glycerol (at a final concentration of 25% vol./vol. glycerol). As frozen strain stocks are frequently accessed, contamination is a threat to stock integrity. Therefore, rigorous aseptic technique is necessary. For example, the use of a biosafety cabinet will greatly reduce the chance of contamination when reviving a frozen stock of bacteria.

Materials

K. pneumoniae frozen stock
Inoculating loop or wooden applicator sticks, sterile (Fisher Scientific, cat. no. 22-029-491)
Luria broth (LB) agar plates (see recipe)

Incubator, static, set to 30°C

1. Using a sterile wooden applicator stick, remove a small chunk of frozen bacteria from the stock vial.

   Freeze-thaw cycles can affect bacterial stock integrity over time. Preparing several stocks in advance and limiting use of a backup stock will help protect stock integrity.

2. Streak the bacteria onto the LB agar plate.

   Starting from the edge of the agar plate, run the inoculated stick back and forth across the plate surface. Using a new sterile applicator stick, gently sweep the tip into the edge of the first quadrant and streak back and forth across the surface of the plate again. Repeat this process, rotating a quarter turn with each streaking, to isolate single bacterial colonies.

3. Incubate the plate overnight for 14 to 18 hr at 30°C or 37°C.

   Many K. pneumoniae isolates grow quickly at 37°C and overgrowth can occur overnight. To avoid colony overgrowth, it is recommended to culture the bacteria at 30°C.

CULTIVATING *K. pneumoniae* IN RICH GROWTH MEDIUM

BASIC PROTOCOL 2

K. pneumoniae can be cultured in a variety of liquid media, each with its own unique composition and applications. Lysogeny broth (LB) is the primary medium used for the cultivation of the bacterium, and the medium can be prepared in three primary

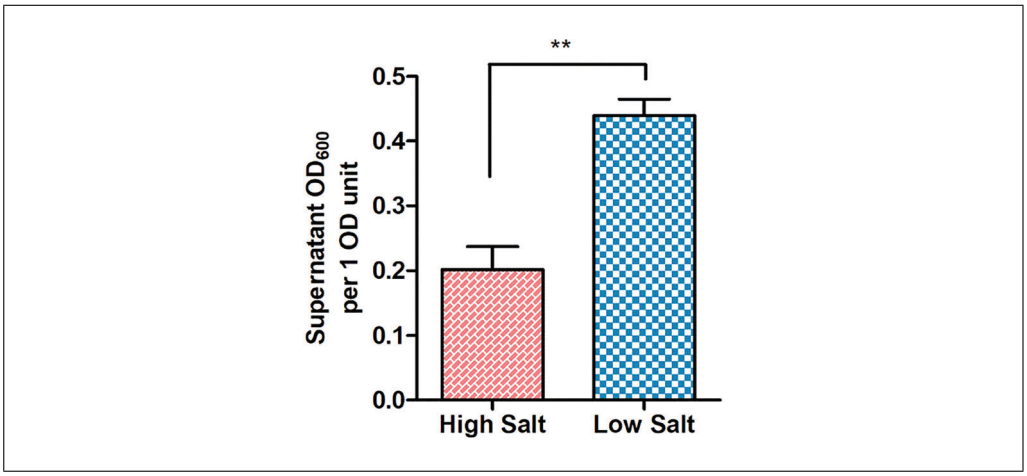

Figure 1 Effect of high-salt versus low-salt conditions on KPPR1 sedimentation resistance. Wild-type KPPR1 was cultured in either high-salt or low-salt LB media overnight, then mucoviscosity was assessed by sedimentation resistance (Khadka et al., 2023b). In brief, 1 OD_{600} of culture was centrifuged for 5 min at 1000 × *g*, room temperature and the OD_{600} of the supernatant was determined. Shown is one independent experiment performed in triplicate, where the error bars represent standard deviation. Significance was determined using a student's t-test, where ** $p < .01$.

Table 2 Common Antibiotics Used with *K. pneumoniae* Strain ATCC 43816[1]

Antibiotic	Solvent	Stock concentration (mg/ml)	Final concentration (μg/ml)
Kanamycin	Water	25	25
Chloramphenicol	Ethanol	80	80
Spectinomycin	Water	50	50
Gentamicin	Water	100	10
Tetracycline	Water	6	6
Apramycin	Water	50	50
Hygromycin	Water	100	100
Rifampin	Methanol	30	30
Streptomycin	Water	500	500

[1] Note that *K. pneumoniae* isolates are ampicillin-resistant.

formulations: Miller, Lennox, and Luria (Sezonov et al., 2007). The Luria formulation is the preferred formulation due to its lower sodium chloride content, which enhances *K. pneumoniae* mucoidy (Fig. 1). See Reagents and Solutions for media recipes.

Each medium can be adapted to the precise requirements for individual experimental conditions. Modifications can be made by incorporating supplements, such as antibiotics at desired concentrations (Table 2), to tailor the different media to suit the specific requirements of the experiment.

Materials

Individual colonies of *K. pneumoniae* on LB agar plate
Luria broth (LB), sterile (see recipe)
Aeration culture tube, sterile, 16-ml, 17 mm diameter (Genesee Scientific, cat. no. 21-131 or equivalent)
Inoculating loop or wooden applicator sticks, sterile (Fisher Scientific, cat. no. 22-029-491 or equivalent)

Shaking incubator, set to 37°C, 200 rpm

1. Using aseptic technique, transfer 3 ml of LB broth to a sterile 16 ml aeration culture tube.
2. Use a sterile inoculating loop or wooden applicator stick to collect a single colony from the LB agar plate.
3. Inoculate the LB broth in the culture tube by agitating the colony on the stick in the LB broth.
4. Replace the cap onto the culture tube to the aerated position.

   It is important to ensure that the cap is in the aerated position to maintain consistent airflow in the tube.

5. Incubate the tube in a 37°C shaking incubator at 200 rpm for 16 to 18 hr, overnight.

   Maintaining a consistent culture angle ensures consistent aeration and improves data reproducibility for down-stream experiments.

CULTIVATING IN MINIMAL LIQUID GROWTH MEDIUM

ALTERNATE PROTOCOL

This protocol is a modified version of Jeffery H. Miller's original work on M9 minimal growth medium (Miller, 1972). It includes updated information on the best practices for cultivating *K. pneumoniae.* In contrast to Basic Protocol 2, the composition of this medium includes a mixture of salts, calcium, magnesium, and can be supplemented with glucose, amino acids, and vitamins, as needed. This variation enables enhanced control over the provided nutrients. The procedure entails preparing a 10× mixture of salts and supplements, performing chelation treatment, sterilizing through filtration, and subsequently introducing metals. By following this protocol, minimal medium enables precise control, reproducibility, and flexibility in the study of organism growth, metabolism, and genetic manipulation. As a result, it serves as a valuable tool in various scientific contexts.

Materials

M9 minimal salts, 10×, sterile (see recipe)
Calcium chloride ($CaCl_2$), 100 mM, sterile (see recipe)
Magnesium sulfate ($MgSO_4$), 1 M, sterile (see recipe)
Glucose, 20% (w/v), sterile (see recipe)
Thiamine, 10 mg/ml, sterile (see recipe)
Casamino acids (CAA), 10% (w/v), sterile (see recipe)
Chelex 100 chelating resin (BioRad, cat. no. 1421253)
Ferrous sulfate ($FeSO_4$), 36 mM in 0.25 N HCl, 36 mM, sterile (see recipe)

1000-ml plastic beaker
Stir bar
Stir plate
Vacuum filter, 0.22 µm (Fisher Scientific, cat. no. S2GPU02RE or equivalent)

Preparing 10× M9 Minimal Salts

1. Make a 1 L volume of 10× M9 minimal salts (see recipe).
2. Autoclave the 10× M9 minimal salts solution to sterilize.

Preparing Stock Solutions

3. Prepare a 100 mM stock solution of $CaCl_2$, 1 M stock solution of $MgSO_4$, and a 20% (w/v) stock solution of glucose (see recipe).
4. Filter sterilize all stock solutions using a sterile vacuum filtration system with 0.22 µm pore size.

Assembling the Medium

5. Add 25 ml of 10× M9 minimal salts and 5 ml of 20% (v/v) glucose, bring the volume up to 250 ml with ultrapure water in a plastic beaker.

   If required, add 5 µg/ml of thiamine and/or 1% (v/v) casamino acids. We do not typically add thiamine and instead use 1% casamino acids, although others have reported using 0.2% casamino acids.

6. Add 0.25 g of Chelex 100 resin (1 g/L) to the solution.
7. Cover the medium with plastic wrap and stir with a stir bar for 3 hr to facilitate metal chelation.
8. Sterilize the solution and remove the Chelex by filtering the medium through a vacuum filter unit with a 0.22 µm membrane under aseptic conditions.
9. Add 0.25 ml of 100 mM $CaCl_2$ stock solution and 0.25 ml of 1 M $MgSO_4$ stock solution to the sterile-filtered medium.

   This is in an iron-depleted condition. If an iron-replete condition is needed, add 36 µM $FeSO_4$ (36 mM stock in 0.25 N HCl) in addition to calcium chloride and magnesium sulfate.

10. Culture *K. pneumoniae* according to Basic Protocol 2 using the prepared M9 minimal medium.

BASIC PROTOCOL 3

ENUMERATING *K. pneumoniae* COLONY FORMING UNITS

The following protocol outlines a method for enumerating *K. pneumoniae* colony forming units (CFUs). Accurate quantification of bacterial CFUs is essential for assessing microbial load and understanding pathogenic potential. This protocol encompasses step-by-step instructions for sample preparation, serial dilution, plating techniques, and incubation conditions to enable the precise enumeration of *K. pneumoniae* colonies.

Materials

K. pneumoniae liquid suspensions
LB agar plates (see recipe)
Phosphate-buffered saline (PBS), sterile (see recipe)

Vortex mixer
96-well, flat-bottom microplates, sterile
Multichannel pipette, 10 to 100 µl
Incubator, static, set to 37°C

Preparing Serial Dilutions

1. Vortex a liquid suspension of *K. pneumoniae* and transfer 100 µl of each sample into row A of a 96-well microplate.

   Replicates may be transferred into separate columns as needed. It is recommended that each sample is prepared in triplicate.

2. Fill rows B to H with 90 µl of sterile PBS.
3. Transfer 10 µl from row A into row B. Mix well by pipetting 10 to 20 times.

   Change pipette tips between each dilution step.

4. Transfer 10 µl from row B into row C. Mix well by pipetting.

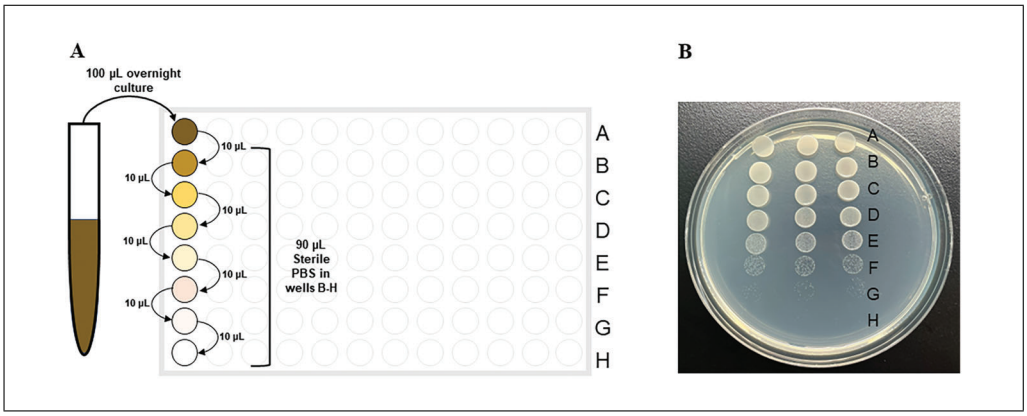

Figure 2 Preparing dilutions for enumerating colony forming units. (**A**) A schematic for preparing dilutions of *K. pneumoniae* within a 96-well microplate. Wells B-H should initially be filled with 90 µl of sterile PBS. 100 µl of liquid culture should be transferred to well A. Serial dilutions of 10 µl from well A to well H should be performed, mixing well and changing pipette tips between each dilution. (**B**) An example spotted dilution of *K. pneumoniae* overnight culture in triplicate on a LB agar plate.

5. Continue this serial dilution series until the final dilution is made in row H, mixing well by pipetting up and down 10 to 20 times and changing pipette tips between each dilution step.

   For a schematic illustration of the serial dilutions, refer to Figure 2A.

6. Using a multichannel pipette, spot 10 µl of each dilution from one column to an individual LB agar plate. Three columns usually fit on a standard LB agar plate.

   Allow LB agar plates to dry on the bench for at least 2 days prior to spot plating. This improves spot absorption into the agar.

   Dispense the 10 µl slowly to avoid dilution spots merging, which prevents accurate colony counting.

   Avoid splattering the dilutions or generating bubbles when pipetting as this can cause CFUs to appear sporadically on the plate.

7. Incubate the LB agar plate with serial dilution spots in a 37°C incubator for 1 to 2 hr and then leave the plate overnight at room temperature.

   The plate may also be left overnight at room temperature and then placed in a 37°C incubator for 1 to 2 hr before counting the colonies. These recommendations are to avoid colony overgrowth and enable accurate counting.

   For an example illustrating the expected colony distribution within each spot, refer to Figure 2B.

Enumerating Colonies

8. Count the colonies at the most diluted spot that can be seen.

   Individual colonies found in the final dilution indicate the presence of $Nx10^9$ colonies in the original suspension. For example, there may be three colonies that can be seen in row H, which would indicate that there were 3×10^9 CFUs/ml within the bacterial suspension.

9. If possible, count the colonies at the second most diluted spot that can be seen.

   For the above example, around 30 colonies should be seen in the spot from row G. This indicates that the serial dilution was performed correctly, diluting each row by a factor of 10.

10. If two countable spots are present, average the number of CFUs counted in each spot.

As CFUs may vary depending on the quality of the serial dilution, averaging the CFU totals from two dilutions provides a more reliable count.

BASIC PROTOCOL 4

GROWTH CURVES

Growth curve analyses provide insight into the growth kinetics and population dynamics of bacterial cultures. Like evaluating growth on solid media, it is important to maintain rigorous aseptic techniques throughout the entire protocol to ensure reliable results. Uninoculated growth medium represents a critical negative control to evaluate aseptic technique. See Understanding Results for sample data and calculations.

Materials

Individual colonies of *K. pneumoniae* on a LB agar plate
LB broth (see recipe)
1× PBS, sterile (see recipe)

Aeration culture tube, sterile, 16-ml, 17 mm diameter (Genesee Scientific, cat. no. 21-131 or equivalent)
1.7-ml disposable cuvettes (Fisher Scientific, cat. no. 14-955-127 or equivalent)
1.7-ml microcentrifuge tubes, sterile
96-well, flat-bottom plates, sterile (Genesee Scientific, #25-104)
Parafilm
Shaking incubator, set to 37°C, 200 rpm
Visible spectrophotometers (for 1.7-ml cuvettes and microplates)

Subculturing

1. Prepare overnight cultures of single *K. pneumoniae* colonies in 3 ml of LB broth as described in Basic Protocol 2.
2. Transfer 3 μl of each overnight culture into three additional 16 ml aeration culture tubes, each filled with 3 ml of LB broth.
3. Incubate in a 37°C, 200 rpm for 5 to 7 hr.

Standardization of Inoculum

4. Pipette 900 μl of PBS into one disposable cuvette per sample.

   Include a cuvette filled with 1000 μl PBS as a blank.

5. Transfer 100 μl of a *K. pneumoniae* sub-culture to the disposable cuvette containing 900 μl PBS. Mix well by pipetting.
6. Measure the optical density of the sub-culture at a wavelength of 600 nm (OD_{600}) using a visible spectrophotometer.

   The OD_{600} of the bacterial sub-culture is 10 × OD_{600} to account for the 1:10 dilution.

7. Calculate the amount of bacterial suspension needed to achieve an initial OD_{600} of 0.0001 in 1 ml of LB broth.

   Be sure to mix the diluted culture thoroughly to ensure homogeneity.

   For example, if the OD_{600} of a sub-culture is 0.1, multiply 0.0001 by 1000 μl and then divide by the OD_{600}. Therefore, the amount of bacterial suspension needed for this instance would be (0.0001 × 1000)/0.1 = 1 μl.

Preparing the Growth Curve Plate

8. Fill 1.7-ml centrifuge tubes with 1 ml of LB broth.
9. Pipette the calculated volume of diluted sub-culture to 0.0001 OD_{600} in each tube.

Table 3 Sample growth curve data from *K. pneumoniae* strain NTUH-K2044

Time (hr)	Replicate 1 (OD_{600})[1]	Replicate 2 (OD_{600})[1]	Replicate 3 (OD_{600})[1]
0	−0.00833	−0.00033	0.013667
0.5	−0.007	−0.004	0.003
1	0.003	0.002	0.013
1.5	0.023333	0.023333	0.026333
2	0.094	0.097	0.093
2.5	0.218333	0.179333	0.193333
3	0.356333	0.262333	0.314333
3.5	0.422333	0.273333	0.350333
4	0.423667	0.285667	0.375667
4.5	0.478	0.314	0.424
5	0.502	0.333	0.403
5.5	0.488333	0.353333	0.455333
6	0.535333	0.366333	0.427333
6.5	0.563333	0.379333	0.454333
7	0.503333	0.394333	0.425333
7.5	0.536333	0.402333	0.462333
8	0.555333	0.407333	0.432333
8.5	0.533333	0.415333	0.440333
9	0.523333	0.423333	0.445333
9.5	0.471333	0.423333	0.441333
10	0.486333	0.433333	0.449333
10.5	0.465333	0.436333	0.453333
11	0.486333	0.443333	0.468333
11.5	0.481333	0.445333	0.469333
12	0.48	0.449	0.475

[1] OD_{600} = Sample OD_{600} – Average Background OD_{600}.

10. Pipette 100 µl from each tube containing 0.0001 OD_{600} of *K. pneumoniae* culture into a well in a sterile 96-well, flat-bottom microplate.

    Include three 100 µl LB blanks in three separate wells.

11. Wrap the edge of the 96-well microplate in laboratory Parafilm.

    This step ensures that the liquid does not evaporate while growth is being monitored. Other measures such as filling the outer wells with PBS, can mitigate the impact of evaporation on results.

12. Load the 96-well microplate into a microplate spectrophotometer.

13. Measure the OD_{600} for the baseline 0 hr timepoint.

14. Run a program that incubates the microplate at 37 °C with orbital shaking (282 cpm) and read the OD_{600} every 30 min for 16 to 18 hr.

    Alternatively, place the microplate in a 37°C shaking incubator and manually measure the OD_{600} every 30 to 60 min on a plate reader.

    Note: faster growing strains may only require 10 to 12 hr to establish stationary phase.

GENOMIC DNA EXTRACTION

This protocol describes a method to extract *K. pneumoniae* genomic DNA using the Wizard® Genomic DNA kit (Promega). The method involves treating cell pellets with cell lysis solution, RNase solution, and proteinase solution to remove contaminants. The extracted DNA is purified and precipitated using isopropanol and ethanol. This protocol provides high-quality and high-yield genomic DNA.

Materials

Individual colonies of *K. pneumoniae* on a LB agar plate
LB broth (see recipe)
Wizard® Genomic DNA purification Kit (Promega, #A1125)
Isopropanol (room temperature)
70% ethanol (room temperature)

Aeration culture tube, sterile, 16-ml, 17 mm diameter (Genesee Scientific, cat. no. 21-131 or equivalent)
1.7-ml microcentrifuge tubes, sterile
Shaking incubator, set to 37°C, 200 rpm
Vortex mixer
80°C heat block or water bath
Microcentrifuge, minimum speed 17,000 × *g*
Clean paper towel

Overnight Culture

1. Inoculate a single, isolated colony of *K. pneumoniae* into 3 ml of LB in a 16-ml aeration culture tube. Incubate at 37°C for 16 to 18 hr with shaking at 200 rpm as described in Basic Protocol 2.

Cell Lysis and RNA Removal

2. Add 1 ml of gently vortexed overnight culture to a 1.7 ml centrifuge tube.

   Before starting the DNA extraction, ensure that the heat block/water bath is set at the required temperature and reagents not supplied in the kit are prepared.

3. Pellet the cells: centrifuge 2 min at 16,000 × *g*, room temperature. Carefully remove the supernatant.

4. Completely resuspend the cell pellet in 600 µl Nuclei Lysis Solution (*provided in the kit*). Ensure that no visible portions of pellet remain.

5. Incubate the mixture at 80°C for 5 min. Then, cool down the cell lysate to room temperature.

6. Add 3 µl of RNase solution (*provided in the kit*) to the cell lysate and mix well by inverting the tube 5 times.

7. Incubate RNase-treated cell lysate for 60 min at 37°C. Then, cool down the RNase-treated cell to room temperature.

Protein Precipitation

8. Add 200 µl of Protein Precipitation Solution (*provided in the kit*) to the tube and mix by vortexing vigorously for 20 sec.

9. Incubate the mixture on ice for 5 min.

10. Centrifuge 3 min at 16,000 × *g*, room temperature.

    Meanwhile, label a new 1.7-ml centrifuge tube and add 600 µl of room temperature isopropanol.

DNA Precipitation and Rehydration

11. Following centrifugation, transfer the supernatant (contains DNA) to the tube containing 600 µl of isopropanol.

    Avoid contaminating the DNA with precipitated proteins. If pipetting up protein precipitate is a concern, then leave about 50 µl of supernatant in the tube.

12. Mix the isopropanol-supernatant solution by gently inverting the tube 6 to 10 times, or until a visible mass of DNA strands is formed.

13. Centrifuge for 2 min at 16,000 × *g*, room temperature.

14. Slowly pour off the supernatant and drain the remaining solution by inverting the tube on a clean paper towel for 10 to 20 sec.

15. Add 600 µl of 70% ethanol (room temperature) to the DNA pellet and invert the tubes 10 to 15 times.

16. Centrifuge the ethanol-washed DNA for 2 min at 16,000 × *g*, room temperature.

17. Gently remove the supernatant using a pipette. Drain the remaining ethanol by inverting the tube on a clean paper towel for 10 to 20 sec.

18. To ensure complete removal of ethanol, allow the tube to air dry for 15 min.

    Air-drying in a biosafety cabinet is recommended.

19. Add 100 µl of DNA Rehydration Solution (*provided in the kit*) to the tube. Resuspend precipitated DNA by incubating at 65°C for 1 hr with periodic mixing of the solution.

    Alternatively, rehydration can be done overnight at room temperature or 4°C.

20. Store rehydrated genomic DNA at −20°C or 2 to 8°C.

CHARACTERIZING *K. pneumoniae* STRAINS BASED ON GENOMIC SEQUENCE

BASIC PROTOCOL 6

Basic Protocol 6 illustrates a guide to characterizing *K. pneumoniae* species complex strains based on genomic sequence. The genomic sequence of a strain of interest is run through several databases and tools, including BIGSdb and Kleborate by Pathogenwatch (Argimón et al., 2021; Lam et al., 2018; Olson et al., 2023; Wyres et al., 2016). Through these established bioinformatic pipelines, a report is generated highlighting key characteristics of the strain based on the genomic sequence. The report generated using this method can be used to characterize *K. pneumoniae* with information such as capsule locus type and lipopolysaccharide (LPS) locus type.

Materials

Desktop or laptop computer with a minimum screen resolution of 1366 × 768 and Chrome, Safari, or Firefox browser
Bacterial and Viral Bioinformatics Resource Centre software, version 3.30.19 (*https://www.bv-brc.org*)
Pathogenwatch software, version 20.5.5 (*https://pathogen.watch*)
Short-reads or assembled whole-genome sequence of *Klebsiella pneumoniae*

Assembly of K. pneumoniae Short-Read Sequence

1. Sign up and log in to BV-BRC (*https://www.bv-brc.org*).

2. At the top of the webpage, click on "Genome Assembly (B)" within the "TOOLS & SERVICES" tab. A new Assembly page will be loaded.

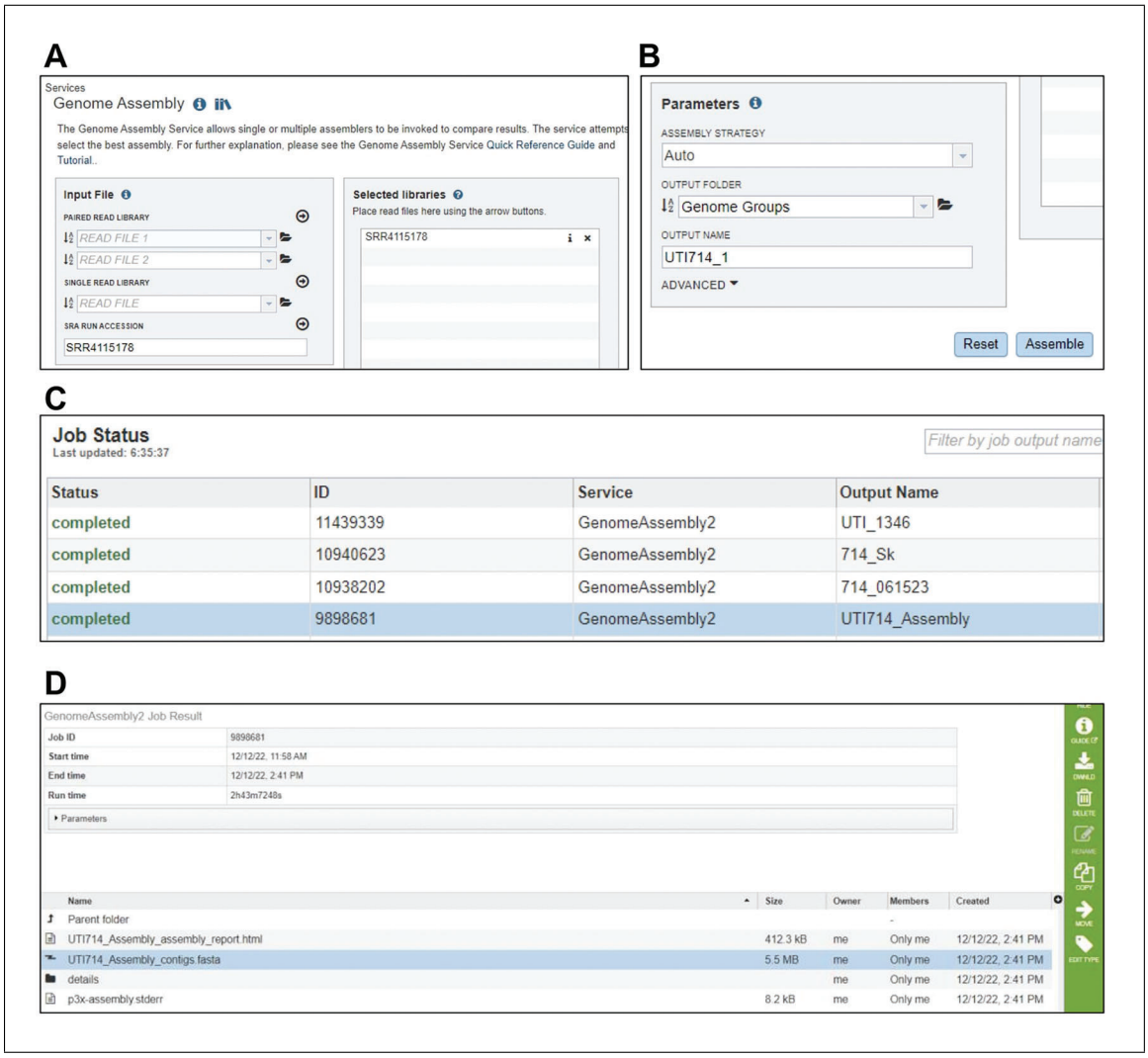

Figure 3 Screen captures of *Klebsiella pneumoniae* clinical isolate genome assembly using BV-BRC. (**A-B**) First, the genome is assembled using BV-BRC. (**A**) The SRA number of *K. pneumoniae* UTI 714 was entered for illustration. (**B**) Analysis strategy is set to 'auto', and output location and folder are defined in the 'Parameters' box. (**C**) Progress of the analysis can be tracked from 'Job Status' box, and the status is changed to 'completed' once the sequence assembly is finished. (**D**) The final assembled sequence can be downloaded as '.fasta' file.

3. If the whole genome sequence of the strain is uploaded in the NCBI Sequence Read Archive (SRA) database, enter the SRA Run Accession number in 'SRA RUN ACCESSION' textbox (Fig. 3A).

   Alternatively, if sequenced data is available offline as a FASTQ file, upload the Single Read Library or Paired Read Library files of the strain. FASTQ files can also be directly used to characterize the strain (go to Step 8).

   SRA Run Accession numbers start with SRRxxxxxxx. Example: SRA Run Accession of Klebsiella pneumoniae isolate 714 is SRR4115178.

4. Click on the adjacent arrow button to upload read files to the "Selected libraries" box. If the input SRA number or read file is valid, the library name will appear in the "Selected libraries" list (Fig. 3B).

5. In the "Parameters" box, select output folder to choose the location for storing analyzed data. Also, type the name of the output file in the "OUTPUT NAME" textbox. Then, click "Assemble" to submit the genome assembly job.

   Based on the genome size and availability of servers, assembly can take several hours.

NOTE: If analyzing multiple K. pneumoniae samples, it is recommended to queue all samples consecutively to improve workflow.

6. In the lower-right corner of the home page, click on "Jobs" to locate the completed assembly. A new page will load.
7. Double click on the completed job. A new window will open containing a list of several files associated with the assembly. Select the file with extension ".fasta" and click on "DWNLD" to download the assembled sequence (Fig. 3C–D).

Characterization of K. pneumoniae using Assembled Whole Genome Sequence

8. Load Pathogenwatch (*https://pathogen.watch*) in a browser and click on "UPLOAD" from the bar at the top. A new page will load.
9. Sign in using one of the available options. Upon signing in, a page to upload sequence files will load.
10. Click on "Single Genome FASTAs". A new page will load.

    Alternatively, select "FASTQ" if sequenced data is available as single or paired read files in FASTQ format.

11. Click on the "+" button at the lower-right corner of the page. Then upload the assembled sequence downloaded from BV-BRC.

    Note: It is recommended to queue all samples consecutively to improve workflow when analyzing multiple data sets. Analysis can take a few minutes to a few hours.

12. Once completed, directly click on "View Genomes" or go to "My Genomes" from the top left menu.
13. From the completed jobs list, open the job with the same file name as the uploaded fasta file. A final report highlighting the species and subspecies of *Klebsiella*, capsule and LPS locus types, virulence-associated genes, and antimicrobial resistance associated genes will load (Fig. 4).

STORAGE OF *K. pneumoniae* FROZEN STOCKS IN GLYCEROL

BASIC PROTOCOL 7

Freezing bacterial cultures as glycerol stocks is a common method for long-term storage and allows preservation of cell viability and genetic integrity. Glycerol serves as a cryoprotectant, minimizing the damage caused by ice crystal formation during the freeze-thaw cycle.

Materials

K. pneumoniae overnight liquid culture
50% glycerol in ultrapure water, sterile

1.8-ml cryovials (Genesee Scientific, #24-203P or equivalent)
Vortex mixer
−80°C freezer

1. Transfer 0.5 ml of an overnight culture into a cryovial containing 0.5 ml of 50% glycerol, vortex well.
2. Label the vial with the strain information and other pertinent information.
3. Transfer to an ultra-low temperature freezer for long-term storage at −80°C.

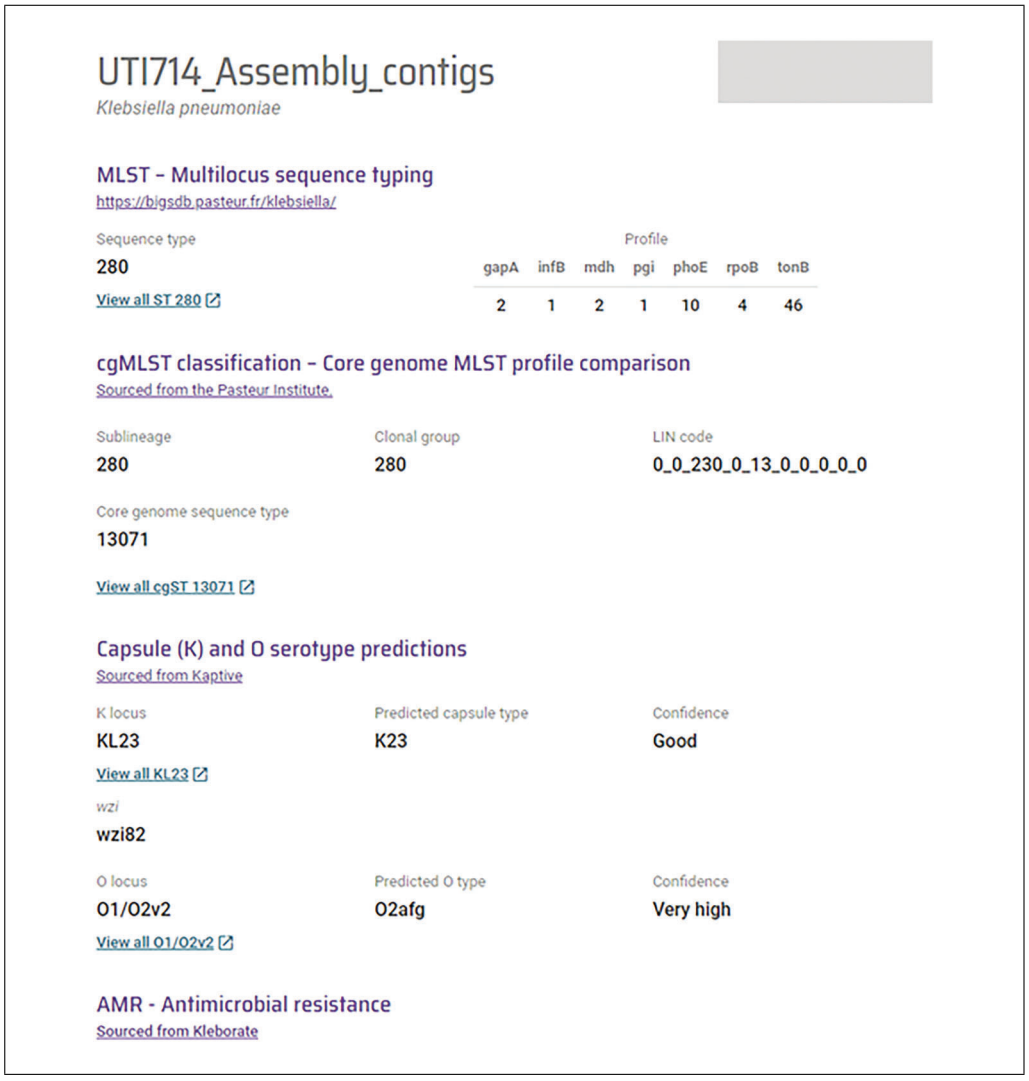

Figure 4 Genome report generated by Pathogenwatch for SRR4115178. The depicted genome is reported to be a *Klebsiella pneumoniae* ST280 isolate with K23 K-type and O2afg O-type.

BASIC PROTOCOL 8

STORAGE OF *K. pneumoniae* IN AGAR STABS

Agar stabs are sometimes used for short-term storage of strains at temperatures above freezing, such as when shipping strains. When shipping strains, it is important to adhere to all federal, state, and institutional guidelines. Please consult with your institutional Environmental Health and Safety Office for a full list of federal, state, and institutional guidelines.

Materials

Individual colonies of *K. pneumoniae* on a LB agar plate or in liquid suspension
Luria broth (LB) agar stabs (see recipe)
Toothpicks, sterile

Incubator, static, set to 37°C

1. Touch a sterile toothpick to an individual colony on a solid media plate, or into an overnight liquid culture of *K. pneumoniae*.
2. Stab almost to the bottom of the vial and then pull the toothpick straight out.

3. Slightly loosen the cap of the agar stab vial and incubate at 37°C for approximately 12 to 18 hr.
4. Screw the cap on tightly and store the vial at 4°C for up to 3 weeks, or room temperature for shipping.

REAGENTS AND SOLUTIONS

Use ultrapure water to prepare all recipes.

Calcium chloride ($CaCl_2$), 100 mM

1.47 g calcium chloride
100 ml chelated water

Dissolve 1.47 g calcium chloride in 100 ml chelated water. Sterilize with a 0.22 μm filter. Store at room temperature for up to one year.

Casamino acids (CAA), 10 % (w/v)

10 g casamino acids
100 ml ultrapure water

Dissolve 10g casamino acids in 100 ml ultrapure water. Sterilize with a 0.22 μm filter. Store at 4˚C for up to 6 months.

Chelated water

1 g Chelex 100 chelating resin (BioRad, cat. no. 1421253)
1000 ml ultrapure water

Dissolve 1 g Chelex 100 chelating resin in 1000 ml ultrapure water with stirring for 3 hours. Remove Chelex 100 by sterile-filtering with a 0.22 μm filter. Store indefinitely at room temperature.

Ferrous sulfate in 0.25 N hydrochloric acid, 36 mM

1 g ferrous sulfate ($FeSO_4$)
100 ml 0.25 N HCl

Dissolve 1g ferrous sulfate in 100 ml of 0.25 N HCl. Sterilize with a 0.22 μm filter. Store at room temperature.

Glucose, 20% (w/v)

20 g glucose
100 ml ultrapure water

Dissolve 20 g glucose in 100 ml ultrapure water. Sterilize with a 0.22 μm filter. Store at 4˚C for up to 6 months.

Luria broth (LB), low-salt

5 g yeast extract
10 g tryptone
0.5 g sodium chloride (NaCl)

Add ingredients and ultrapure water to a final volume of 1000 ml in a 4-L beaker. Stir until all solids are dissolved, then aliquot into clean glass bottles. Autoclave the media bottles with the lids slightly loosened.

Store at room temperature up to 6 months.

Luria broth (LB) agar

5 g yeast extract
10 g tryptone

0.5 g sodium chloride (NaCl)
20 g agar

Add ingredients and ultrapure water to a final volume of 1000 ml in a 2-L flask. Stir while heating until all solids are dissolved. Autoclave to sterilize. Allow the flask to cool enough to handle without heat-protective gloves and then add antibiotics, as necessary.

To pour agar plates: pour ∼25 ml into sterile 100 mm × 15 mm Petri dishes. Allow to solidify at room temperature. Dry at room temperature for 1 to 2 days. Store in sealed bags at 4°C for up to 3 months.

To make agar stabs: Aliquot 1 ml warm LB agar into a 1.8 ml cryotube. Close the lid loosely and allow the agar to solidify at an angle. Store at 4°C for up to 3 months.

If antibiotics are added, it may be necessary to store the plates protected from light.

M9 minimal salts, 10×

60 g sodium dihydrogen phosphate (Na_2HPO_4)
30 g monopotassium phosphate (KH_2PO_4)
5 g sodium chloride (NaCl)
10 g ammonium chloride (NH_4Cl)
1000 ml ultrapure water

Dissolve salts in ultrapure water and bring to 1000 ml. Autoclave and store indefinitely at room temperature.

Magnesium sulfate ($MgSO_4$), 1 M

12.03 g magnesium sulfate
100 ml chelated water

Dissolve 12.03 g magnesium sulfate in 100 ml chelated water. Sterilize with a 0.22 μm filter. Store at room temperature for up to one year.

Phosphate-buffered saline (PBS), 10×

80 g sodium chloride (NaCl)
2 g potassium chloride (KCl)
14.4 g sodium dihydrogen phosphate (Na_2HPO_4)
2.4 g monopotassium phosphate (KH_2PO_4)

Dissolve salts in 800 ml ultrapure water. Adjust pH to 7.4 with sodium hydroxide (NaOH). Add ultrapure water to 1 L total. Autoclave and store indefinitely at room temperature.

Thiamine stock solution, 10 mg/ml

100 mg thiamine
10 ml ultrapure water

Dissolve 100 mg thiamine in 10 ml of ultrapure water. Sterilize with a 0.22 μm filter. Store at 4°C for up to 2 weeks.

COMMENTARY

Background Information

K. pneumoniae has emerged as a significant global health concern in recent decades. The bacterium can infect a wide range of tissues and rapidly develop resistance to antibiotics, making it considerably potent in the hospital setting. Traditionally, the classical *K. pneumoniae* (cKp) strains are commonly known to be opportunistic pathogens, affecting those with comorbidities and the immunocompromised. However, the rise of hypervirulent strains of *K. pneumoniae* (hvKp) has allowed the bacterium to cause infections in young and healthy individuals without traditional risk factors. These strains are frequently associated with serious infections such as liver abscesses, meningitis, and endophthalmitis (Russo & Marr, 2019).

Table 4 Troubleshooting Guide for Reviving *K. pneumoniae* from Frozen Stocks

Problem	Possible cause	Solution
No visible growth	Insufficient bacteria transferred from stock vial	Retry streaking process with a larger portion of bacteria-containing ice from the frozen stock
	Improper agar medium and/or supplements used	Ensure that a rich agar medium is used, such as LB, and all correct supplements, such as antibiotics, were introduced into the agar medium at the correct concentration
	Viability of the bacteria in the frozen stock has decreased	Plate the entire frozen stock and make another frozen stock vial
Growth in the negative control	Growth medium is contaminated	Prepare a new batch of growth medium for use

Table 5 Troubleshooting Guide for Growth Curve Analyses

Problem	Possible cause	Solution
Insufficient growth	Inoculum size is too small	Increasing the inoculum size to an OD_{600} above 0.0001, such as 0.001 or 0.01
	Temperature fluctuations within the microplate reader	Ensure that the microplate reader is maintaining a constant temperature of 37°C for the entire duration of data measurement
Growth in the negative control	Growth medium is contaminated	Prepare a new growth curve with new growth medium

Although there is a rising threat and prevalence of the hvKp strains, there are still knowledge gaps in understanding host-pathogen interactions, virulence determinants, and pathogenesis within different tissue types. The cultivation of laboratory strains and extraction of genomic DNA for study are essential first steps to developing a working understanding of *K. pneumoniae* pathogenesis mechanisms.

Critical Parameters and Troubleshooting

The quality of the final report generated by Basic Protocol 6 depends on the input genomic sequence. It is critical to ensure that the correct genomic sequence (short-reads or SRR) is submitted to BV-BRC for assembly. Based on the sequence read quality, the platform may fail to generate coherent contigs, ultimately affecting analysis by Pathogenwatch. Pathogenwatch may not be able to identify capsule or lipopolysaccharide type in the final report if it is a novel K- or O-type yet to be updated in the database. Further, it is important to ensure that the most recent versions of both programs are used for analysis. Troubleshooting guides for reviving *K. pneumoniae* from frozen stocks and growth curve analysis can be found in Tables 4 and 5.

Statistical Analysis

Basic Protocol 4 describes the generation of growth curves. From the data obtained in this experiment, the doubling time can be calculated by identifying an early (t_1 and OD_1) and a late (t_2 and OD_2) time point within the most linear portion of the logarithmic growth phase and then applying the following equation.

$$\text{doubling time (in minutes)} = 60\,x \left[\frac{\ln(2)}{\ln\left(\frac{\frac{OD_2}{OD_1}}{t_2 - t_1}\right)} \right]$$

Equation 1

If utilizing GraphPad Prism (version 8.3.0 or later) for data analysis, the area under the curve can also be calculated to quantify the overall growth of the strain (Mike et al., 2021).

Understanding Results

Basic Protocol 1 describes the revival of *K. pneumoniae* from a frozen stock culture. After streaking and overnight incubation in 37°C, single, isolated colonies should be visible away from the initial streaking area and should be large, round, and white in color.

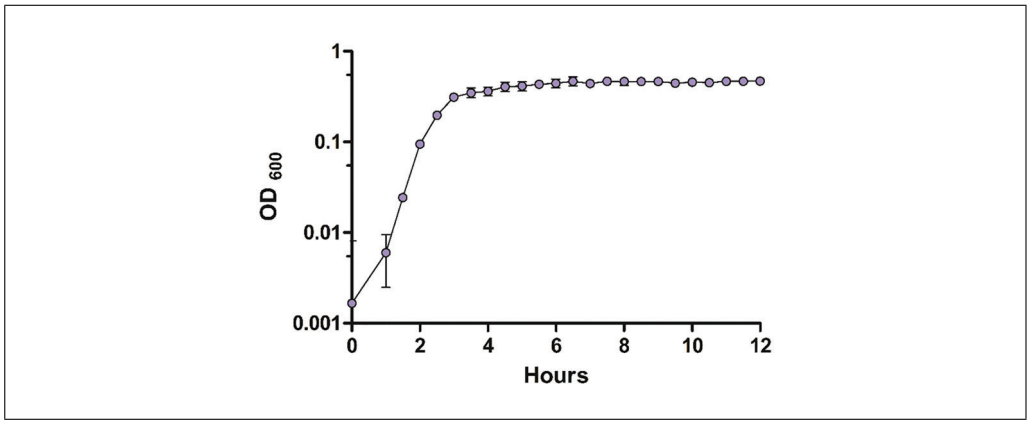

Figure 5 Sample growth curve of *K.pneumoniae* strain NTUH-K2044. NTUH-K2044 was cultured in LB and the OD_{600} was recorded every 30 min for 12 hr. The values plotted have the background value subtracted from the culture OD_{600} (Table 3). The graph illustrates the growth of the bacterial population from the lag phase to the exponential phase, followed by the stationary phase. The decline in growth during the late stationary phase was not seen in this experiment but may be observed in longer periods of monitoring.

Basic Protocol 2 should yield a turbid liquid culture with no significant change in medium color. Basic Protocol 3 describes counting CFUs on an LB agar plate. After 37°C incubation, single colonies should only be present where the plates were spotted. Optimal growth is achieved when two of the most dilute spots have distinct, individual colonies.

Basic Protocol 4 describes the generation of growth curves over a period of time. An expected OD_{600} data set may look like the data generated in Table 3. The values shown have the background value subtracted from the culture OD_{600}. This data set illustrates three samples of the NTUH-K2044 laboratory strain over a period of 12 hr. 16 to 18 hrs is recommended when conducting growth curves for the first time. The time course for subsequent experiments may be adjusted for fast or slow-growing strains. A growth curve can then be plotted from this data. An example of a curve generated from the data in Table 3 is shown in Figure 5. Note that the decline in growth during the late stationary phase may not always be seen, such as in this experiment. Longer monitoring may be necessary to see every phase.

Basic Protocol 5 describes the extraction of genomic DNA from a *K. pneumoniae* culture. The anticipated result is the production of a genomic DNA stock that can be used for downstream experiments. Basic Protocol 6 describes characterizing *K. pneumoniae* strains based on genomic sequence. A genome report (Fig. 4) with detailed information about the genus and species of the organism, sequency type, lineage, capsule and LPS type, antimicrobial resistance profile, genome clustering and plasmids is anticipated upon successful completion of Basic Protocol 1. Serotype predictions are provided with 'confidence' level. Generally, serotype reports with 'good' or higher confidence are desirable.

Basic Protocol 7 describes the storage of *K. pneumoniae* in glycerol as frozen stocks. The anticipated result is the production of frozen stocks for long-term use and extended maintenance of bacterial integrity. Basic Protocol 8 is a similar method that produces more short-term storage of *K. pneumoniae* agar stabs with the additional benefits of being stable at room temperature and thus easily shippable.

Time Considerations

Preparing input bacterial samples for a growth curve requires approximately 24 hr of mostly incubation time. Depending on the number of samples, subculturing for growth curves (Basic Protocol 4) can be completed in 5 to 15 min. Measuring and calculating the inoculation size to achieve an optical density of 0.0001 can be completed in 10 to 20 min. Preparing the 96-well microplate with 100 µl of each sample can be completed in 10 to 15 min. Not including culture of the input bacteria and the 16 to 18 hr data collection, the preparation of the bacteria for growth curves should take 25 to 50 min.

When extracting genomic DNA (Basic Protocol 5), allow 16 to 18 hr for the bacteria to grow overnight. Incubating the RNase-added cell lysate at 37°C will take 60 min and resuspending the precipitated DNA at 65°C will take an additional 60 min. Several shorter incubation periods total approximately 25 min, and several centrifugation steps total approximately 9 min. To remove the ethanol from the

DNA pellet, it is recommended to air-dry the tube for 15 min. Basic Protocol 5 should take about 2.5 to 3 hr, not including the overnight culture.

In Basic Protocol 6, the genome assembly process within the BV-BRC online applet may take several hours depending on genome size and the availability of servers. The analysis of whole genome sequences within Pathogenwatch may take a few minutes to an hour depending on server availability.

The remaining Basic and Alternate protocols should take no more than few minutes each, not including the time required to grow the bacteria overnight (e.g., 16 to 18 hr). With larger sample sets, additional time should be allotted to handle multiple samples.

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Author Contributions

Drew Pariseau: Conceptualization; data curation; methodology; supervision; writing—original draft; writing—review and editing. **Brooke Ring:** Writing—original draft; writing—review and editing. **Saroj Khadka:** Writing—original draft; writing—review and editing. **Laura Mike:** Conceptualization; methodology; project administration; supervision; writing—original draft; writing—review and editing.

Conflict of Interest

The authors declare no conflicts of interest.

Data Availability Statement

Data sharing is not applicable to this article as no new data were created or analyzed in this study.

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