Modified Pulse-Net Procedure for Pulsed-field Gel Electrophoresis of Select Gram Negative Bacilli

Purpose

The purpose of this procedure is to outline the protocol for performing molecular subtyping using pulsed-field gel electrophoresis (PFGE) for select enteric and non-fermentative Gram negative bacilli (shown in Appendix B) received from outbreaks and surveillance studies by the Division of Healthcare Quality Promotion, Centers for Disease Control and Prevention

Scope

This document applies to the laboratory staff performing molecular subtyping of select enteric and non-fermentative Gram negative bacilli.

Responsibility

Role	Responsibility
Laboratory staff	Follow procedure as directed.

Related Documents

Title	Document Control Number
PFGE Worksheet	ENV.TE.C.0012.F01
Maintenance of the CHEF Pulsed Field Gel Electrophoresis Rig	CEM.EQ.C.0002

Definitions

BSA	Bovine serum albumin
CHEF	Contour-clamped homogeneous electric field
PFGE	Pulsed-field gel electrophoresis
EDTA	Ethylenediaminetetraacetic acid
TE Buffer	Tris-EDTA buffer (0.01M, pH8.0)
TBE Buffer	10X Tris-Borate EDTA buffer
TIFF	Tagged Image File format
RO water	Reverse osmosis water
BAP	Trypticase Soy Agar II with 5% sheep blood

References

1. Manual of Clinical microbiology, 10th Edition, Chapters 37, 40, 41, and 42
2. CHEF Mapper® XA Pulsed-Field Electrophoresis System, Instruction manual and Application Guide, Catalog Numbers 170-3670 to 170-3673

Sample information / processing

- All isolates for PFGE should be processed from pure cultures from a BAP.
- Check reference for optimal growth temperature for the particular organism and incubate accordingly.

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Materials

Equipment	Reagents & Media	Supplies
• Bio-Rad Pulsed field Electrophoresis System with cooling module and variable speed pump • Casting stand with frame and platform • Comb holder and 15 well comb • Bubble level • Incubators (25°C, 30°C, 37°C) • 65°C waterbath • 55°C waterbath • Analytical balance • Microwave oven • Tube racks for microcentrifuge tubes and for 17 x 100 mm tubes • Microliter pipetters, 10 µL, 20 µL, 200 µL, 1000 µL. • Reusable plug molds • -20°C freezer • Single edge razor blade and holder • Vortex mixer • Table-top rotator • Refrigerator (4°C) • Cutting platform • MicroScan turbidity meter • Leveling platform • Lead rings (small and medium • Shaker-incubator	• Trypticase Soy Agar II with 5% sheep blood • TE Buffer, pH 8.0, 0.01M • TBE buffer, 10X • Thiourea • Proteinase K solution (20mg/mL) • SeaKem® Gold Agarose • Gel Red nucleic acid stain or Ethidium Bromide • Sterile distilled water • Reverse Osmosis (RO) water • *Salmonella* ser. Braenderup H9812 (Global standard) • 3% Amphyl solution • 70% Isopropyl alcohol • *Xba*I restriction enzyme and Buffer D • *Spe*I restriction enzyme and NEB 4 Buffer • *Apa*I restriction enzyme and Buffer A • Pulse Net Gram Negative Suspension Buffer (see Appendix A) • Pulse Net Gram Negative Lysis Buffer (See Appendix A) • EDTA • 1M Tris-HCl • N-Lauroylsarcosine	• Sterile transfer loops • 1.5 mL sterile microfuge tubes • Sterile pipette tips for P10, P20, P200, and P1000. • 50 mL Erlenmeyer flasks • 125 mL Erlenmeyer flasks • 500 mL Erlenmeyer flask • Latex or nitrile gloves • 2000 mL cylinder • 100 mL cylinder • Black Sharpie Fine point permanent marker • 25 cm x 25 cm gel staining tray with lid • Kimwipes, small and large • 17 x 100 mm tubes with caps • Ball point pen • Weighing paper • 800-1000 mL disposable discard beakers • 25 mL sterile pipettes • Teflon-coated or stainless steel spatula • Spare electrodes • 0.2 µ disposable filter unit • Sterile bottles with screw caps (500 mL and 1 L)

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Safety Precautions
All procedures should be performed under BSL-2 conditions as outlined in the Laboratory Biosafety Manual unless otherwise specified for that particular organism.

Quality control
As performed by CDC Scientific Resources Program and associated manufacturers. The Global Standard, *Salmonella* ser. Braenderup H9812, is added to the end wells of each gel and additionally between every 4 to 6 samples. When new Salmonella Standard is prepared, it must be run and compared with the previous Salmonella Standard to insure Salmonella Standard integrity.

Examination Procedures:

Preparation of Agarose Plugs	
Step	**Action**
1.	Turn on the shaker/incubator to 54°C
2.	Turn on the 55° and 65°C water baths.
3.	Combine sufficient SeaKem Gold Agarose and TE Buffer in a 125 mL Erlenmeyer flask to prepare a 1% solution. Dissolve agarose by heating gently in the microwave. Pour agarose solution into a 50 mL Erlenmeyer flask, stabilize the flask with a small lead ring, and place in the 65°C water bath to equilibrate.
4.	Label BAPs of overnight isolate test cultures.
5.	Label one 17 x 100 mm tube for each culture plate.
6.	Transfer 2 mL of Pulse-Net Gram-negative Cell Suspension Buffer to each 17 x 100 mm tube. (See Appendix A for composition of Cell suspension Buffer and Pulse-Net Gram-Negative Lysis Buffer)
7.	Using a disposable culture loop, add sufficient organism to the Suspension Buffer to give an absorbance reading of 0.55 to 0.60 in the MicroScan Turbidity meter. Adjust as needed with the Cell Suspension Buffer.
8.	Label a 1.5 mL microcentrifuge tube for each culture being tested.
9.	Transfer 200 µL of the cell suspension to the appropriately labeled microcentrifuge tube.
10.	Add 10 µL of Proteinase K Solution to each microcentrifuge tube.
11.	Label reusable plug molds.
12.	Add 200 µL of 1% SeaKem solution to a microcentrifuge tube containing the cell suspension/ Proteinase K, mix gently by pipetting up and down using the pipette tip, and then dispense into a well of the plug mold avoiding air bubbles.
13.	Allow plugs to solidify in the refrigerator for at least 15 minutes.
14.	Prepare a solution of Pulse-Net Gram-Negative Lysis Buffer supplemented with 0.1 mg/mL Proteinase K sufficient to add 5 mL to a 17 x 100 mm tube for each isolate. Label each tube with the culture number, the date, and the name of the organism being tested.
15.	Using a spatula, carefully remove a plug from the plug mold and place it into the properly labeled tube of Lysis Buffer.
16.	Place tubes in a rack in the shaker-incubator with temperature set at 54°C and 50 RPM for 2 h.

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Preparation of Agarose Plugs	
Step	**Action**
17.	Pre-heat sufficient distilled water to 55°C to wash plugs 2 times with 10 mL of heated distilled water.
18.	After 2 h, remove tubes from 54°C shaker-incubator and adjust shaker- incubator to 50°C.
19.	Carefully pour off Cell Lysis Buffer and blot tube on Kay Dry to remove residual Lysis Buffer (blotting is optional).
20.	Add 10 mL heated distilled water to each tube.
21.	Shake tubes gently at 50 RPM in 50°C shaker-incubator for 15 minutes.
22.	Repeat preheated distilled water wash step once more.
23.	Preheat TE Buffer at 55°C.
24.	After the two distilled water wash steps, pour off the water and blot as before.
25.	Wash each plug with 10 mL preheated TE Buffer for 15 minutes four times in a shaker-incubator set at 50°C and 50 RPM.
26.	Decant and blot (blotting is optional) after the last wash, then add 5 mL of TE Buffer to cover the plug.
27.	Store plugs at 4°C until ready to use.

Restriction Digest of DNA in Agarose plugs	
Step	**Action**
1.	Label a 1.5 mL microcentrifuge tube for each sample to be run.
2.	Prepare sufficient Master Mix containing the following for each sample: 135 µL Type 1 water 15 µL specific buffer according to restriction enzyme used 3 µL bovine serum albumin 5 µL restriction enzyme Finger vortex mixture. Dispense 158 µL into each microfuge tube.
3.	Remove plugs from refrigerator. Use a single edged razor blade to remove the rounded corners of the agar plug. Then cut a thin slice of the plug for testing. The length of the slice will depend on the comb size to be used. For the 15 place comb, the slice should be about 9 mm long.
4.	Place the agarose slice into a 1.5 mL microcentrifuge tube containing the Master Mix.
5.	Cut the same size slices of the Salmonella Braenderup Standard, H9812. These are always restricted with *Xba* I and the appropriate buffer (see Master Mix formula). Cut at least three Standards for the 15 place comb.
6.	Incubate the slices to be restricted at the appropriate time and temperature recommended by the manufacturer for the particular restriction enzyme. Time can vary from 2 to 4 h or overnight.

Casting the Agarose Gel	
Step	**Action**
1.	Prepare 2000 mL of 0.5X TBE buffer by adding 1900 mL of RO water to 100 mL of 10X TBE buffer.

2.	Using the analytical balance, weigh 1.5g of SeaKem Gold Agarose and place into a 500 mL sterile Erlenmeyer flask.
3.	Add 150 mL of the 0.5X TBE buffer to the agarose. Microwave the mixture to dissolve the agarose, and place the mixture in a 55°C waterbath to equilibrate for at least 20 minutes.
4.	Assemble the platform in the casting stand so that it slides into the grooves at each of the short ends of the casting stand frame. Place the casting stand on the leveling platform. Check that the system is level by using the leveling bubble and adjust as necessary.
5.	Rest the comb holder that is holding the comb on the casting stand, and load the restricted plug slices evenly on the ends of the comb wells including Standards as needed. A tiny amount of melted agarose can be added to the backs of the plug slices to stabilize them on the comb.
6.	Place each side of the comb holder into the positioning slots on each side of the casting stand. Check that the bottom of the comb is about 2 mm above the surface of the platform.
7.	Pour the 150 mL of melted agarose into the casting stand, and let solidify for 30 minutes.
8.	Level the electrophoresis chamber by adjusting the leveling screws. Check with the leveling bubble.
9.	[If using thiourea, add 760 µL of thiourea to the running buffer.] Pour the 0.5X TBE buffer into the electrophoresis chamber, and turn on the pump switch. After making certain that the buffer is flowing through all the tubing, turn on the chiller and set the temperature at 14°C. **(Addition of thiourea is optional)**
10.	Using the pins on the casting frame, stabilize it using the holes on the floor of the chamber.
11.	When the buffer in the chamber has reached 14°C, remove the comb from the agar in the casting stand. Carefully remove the gel from the casting stand by removing the end pieces from the casting stand so that only the gel remains on the platform. Inspect the gel on the platform and gently remove any gel "tag" from the sides and bottom of the platform using a Kimwipe. Place the gel into the frame in the chamber.

Electrophoresis Conditions	
Step	**Action**
1.	CHEF Mapper® XA Pulsed Field Electrophoresis System: • Follow operation/setup procedures as described in Instruction Manual and Application Guide. • Select the following conditions for "Two State": • Gradient: 6V/cm (i.e. 200V) • Included angle: 120 • Ramping factor: Linear • Start and Final Switch times (in seconds) • Run time (in hours) CHEF DRII: • Turn on the power supply after securing the lid onto the rig. • Press "Volts/CM" • Raise to 6.0 (6V/CM=200V)

Electrophoresis Conditions	
Step	**Action**
	• Press "Run Time" (HRS) • Raise to length of gel run • Press "Block" and "Volts/CM" simultaneously • Raise to the initial ramp or switch time • Press "Volt/CM" and "Run time" simultaneously • Raise to set final ramp time • Press "Start/Pause" to begin gel run
2.	Set appropriate switch times and gel run time for the organism being tested (see Appendix B).
3.	Start run. Ensure that all electrodes are functioning (bubbles are generated during ionization).
4.	Document the following on the PFGE Worksheet (see Appendix C) • Genus and species of the organism • Date of plug preparation • Date of digest and incubation temperature • Rig # used • Run conditions (switch times and run time) • Gel type and concentration • Gel size • Buffer temperature during run • Voltage • Amount of thiourea used • Assign lane numbers for each specimen and each Standard • Lot numbers of all reagents

Staining and Imaging the Gel	
Step	**Action**
1.	After the PFGE run is completed, turn off the cooling module. Let the pump run for at least 15 minutes to avoid freezing up the cooling apparatus.
2.	Stain the gel in a solution of 300 mL RO water and 30 µL of Gel Red Nucleic Acid Stain. [Alternately 25 µL ethidium bromide (EtBr) and 500 mL of RO water may be used]. Add the solution to a plastic tray. Slide the gel carefully into the solution while removing the platform underneath the gel. Place the gel onto a table-top rotator and rotate gently for 30-45 mins. (If using EtBr, destain in 1L of RO water. Cover the plastic tray with foil and shake gently for 1 h).
3.	Capture the DNA image in the gel using UV light and an imaging system. Save to a tiff file for Bionumerics analysis. Make a photo of the gel using the digital camera. Attach photo to worksheet.
4.	Discard gel into an autoclave waste pan. Wash plastic tray with warm tap water, rinse with RO water, and allow to air dry.

Method Performance Specifications
Cleaning and Maintenance of the Rig -See separate document: Maintenance of the CHEF Pulsed Field Gel Electrophoresis Rig CEM.EQ.C.0002

Calculations
N/A

Reference Intervals, Alert Values
N/A

Interpretation of Results
Gels are analyzed using Bionumerics software (Applied Maths, Austin, TX).

Reporting; guidelines for notification
N/A

Revision History

Revision #	Control #	Changes Made to Document Section	Author	Date
00	N/A	New Document	PulseNet USA	2007
01	N/A	1. Use 200 µL cell suspension 2. Use 10 µL Proteinase K 3. Shake at 50 rpm. 4. Total volume of buffer plus water per plug slice is 150 µL. 5. Incubate samples and control plug slices for 4 h or overnight with restriction enzyme.	Bette J. Jensen	2010
02	2013-17	1. Convert to QMS format 2. Optional use of Gel Red for staining gel with no rinsing needed.	Bette J. Jensen	1/23/2014

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Approval Signatures

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Attachments/Appendices

A. Reagent preparation
B. Restriction enzymes, switch times, and run times for selected Gram negative bacilli
C. Example of Pulsed Field Gel Electrophoresis Worksheet

Appendix A: Reagent preparation

PulseNet USA (Gram negative) Cell Suspension Buffer

For 1000 mL of solution: 100 mL 1M Tris, pH 8.0
200 mL 0.5M EDTA

1. Add Tris and EDTA to a 1000 mL cylinder
2. Add RO water to dilute to 1000 mL.
3. Filter sterilize using a 0.2 µ disposable filter or autoclave the solution.
4. Transfer to a sterile (Wheaton) bottle.

PulseNet USA (Gram negative) Cell Lysis Buffer

For 1000 mL of solution: 50 mL 1M Tris, pH 8.0
100 mL 0.5 M EDTA, pH 8.0
10 g N-Lauroylsarcosine, Sodium salt

1. Mix well in a 1000 mL cylinder.
2. Add RO water to dilute to 1000 mL.
3. Filter sterilize using a 0.2 µ disposable filter unit.
4. Transfer to a sterile (Wheaton) bottle.

Appendix B: Restriction enzymes, switch times, and run times for selected Gram negative bacilli

Organism	Restriction enzyme	Switch time (seconds)	Run time (hours)
Acinetobacter baumanii complex	*Apa* I	7 and 20	18.2
Burkholderia cepacia complex	*Spe* I	2 and 50	22
Citrobacter koseri	*Xba* I	5 and 40	22
Enterobacter cloacae	*Xba* I	5 and 40	21
Enterobacter sakazakii	*Xba* I or *Spe* I	1.8 and 25	20
Escherichia coli	*Xba* I	2 and 40 or 5 and 40	22
Klebsiella oxytoca	*Xba* I	2.2 and 64	18
Klebsiella pneumoniae	*Xba* I	5 and 40	22
Morganella morganii	*Xba* I	2 and 50	20
Proteus mirabilis	*Xba* I or *Apa* I	2 and 50	20
Providencia rettgeri	*Apa* I	2 and 40	22
Providencia stuartii	*Xba* I	2 and 40	22
Pseudomonas aeruginosa	*Spe* I	5 and 40	21
Pseudomonas fluorescens	*Spe* I	10 and 30	22
Serratia marcescens	*Spe* I	2 and 40	22

Appendix C: Example of Pulsed Field Gel Electrophoresis Worksheet

PFGE Operator: _____

Organism: _____

Outbreak/ Study#: _____

Rig#: _____

Date of gel run: _____

Date of gel digest: _____

Date of plug prep: _____

Restriction enzyme_____

Enzyme amount _____ **µL/plug**

Buffer_____

Lane:

1. _____

2. _____

3. _____

4. _____

5. _____

6. _____

7. _____

8. _____

9. _____

10. _____

11. _____

12. _____

13. _____

14. _____

15. _____

Lot # _____

Buffer amount _____ **µL/plug**

Temp: _____**37^{0}C**_____**25^{0}C**

Restriction time in: _____**out:** _____

Agarose: SeaKem Gold

Lot # _____

Concentration: _____**%**

Buffer: 0.5X TBE; Other: _____

Lot # _____

Thiourea in running buffer_____**mL**

Size: 21 X 14 cm; Other: _____**cm**

Run Conditions: Voltage _____

Pulse interval (sec.):

Initial _____ **Final** _____

Length of run (hr.):_____

Temp: Initial _____ **^{0}C Final** _____ **^{0}C**

Gel run time: On _____ **Off** _____