DIAGNOSIS OF SEGMENTED GENOME VIRUSES WITH POLYACRYLAMIDE ELECTROPHORESIS (PAGE)

Majority of the viruses are consisted of monopartite genomes, whereas segmentation is also a much more common phenomena seen in RNA viruses. In a group of viruses (all RNA viruses), the linear genome splits into two or more segments. The RNA viruses with genetic material have segmented genomes are influenza viruses, birnaviruses, picobirnaviruses, arenaviruses, bunyaviruses and rotaviruses. Here, we have used rotavirus to address the question of diagnosis of segmented viruses using its segmented genome based methodology. The rotavirus belongs to the *Reoviridae* family and is a major cause of acute gastroenteritis in animal and human neonates. Estimates shows that as many as 600,000 children die every year from rotavirus infection with more than 80% of the deaths occurring in developing countries of South Asia and sub-Saharan Africa. This virus is icosahedral, non-enveloped, triple-layered particle (TLP) and contains genome of 11 segments of double-stranded RNA (dsRNA). It can be detected in clinical specimens by extraction of the viral RNA and analysis by electrophoresis on a polyacrylamide gel followed by silver staining. During electrophoresis through the gel, these negatively charged macromolecules separate according to their size. The patterns of dsRNA can be visualized in the gel after staining with silver nitrate. After staining, the gels can be dried and stored.

Requirements for PAGE and silver staining

Micropipettes, Vortex mixer, Water bath (variable temperature), Microfuge, Refrigerator, Electrophoresis apparatus (gel assembly and electrophoresis tank), Power pack, Laboratory scale, spatula, and weighing boats, Plastic/glass dishes, Timer, Orbital rotator, Gel dryer (vacuum or air), Eppendorf tubes, Yellow/blue tips, Marker pen, Distilled water, PAGE and silver staining reagents like Acrylamide/Bisacrylamide (29.2:0.8%), Ammonium per sulphate 10%, TEMED, 0.5M TE Buffer (6.8 PH), 1.5M TE Buffer (8.8 PH), 5x Tris-Glycine buffer, 2X gel loading dye and Ethidium bromide (Stock 20 mg/ml)

Protocols

1. Phenol-chloroform method of RNA extraction from diarrhoeic stool

i. Take 0.5 mL of a 10% stool suspension (prepared in PBS) into aneppendorf tube and add 50µl of a pre-warmed solution of 1 M sodium acetate with 1% SDS.
ii. Vortex for 10 sec and incubate at 37°C for 15 min.
iii. Add an equal volume of phenol-chloroform, vortex for 1 min, and incubate for an additional 15 min at 56°C.
iv. Open and immediately reseal the tubes before further vortexing.
v. Vortex for 1 min and then centrifuge at 12,000 rpm for 3 min and transfer the upper aqueous phase to a fresh tube.
vi. Add 0.25 mL of phenol-chloroform to the solution and repeat steps 5-8 (phenol extraction).
vii. To this, add 1/10 volume (~40µl) of 3M sodium acetate (pH 5.0) and 0.7 mL of ice-cold absolute ethanol. Mix gently by inversion 4-6 times, and incubate at -2°C for 2 hrs and at -7°C for 30 min.
viii. Centrifuge at 12,000 rpm for 15 min at 4°C. Decant the ethanol immediately, and invert the tube onto a paper towel to dry for more than 15 min.
ix. Using the pipette, re-suspend the RNA pellet in 30µl of RNA loading buffer.

2. Polyacrylamide gel electrophoresis protocol

i. Clean the glass plates with detergent and water before final wipe with 70% ethanol. Keep the plates till ethanol evaporates completely.
ii. Assemble the glass plates for gel casting. Mark the top level of the resolving gel on the plate with a arker pen, remembering to leave room for the stacking gel above the resolving gel.
iii. Prepare the 10% resolving gel as given in appendix. Pipette the acrylamide-bisacrylamide solution between the glass plates to the mark and overlay the gel with a layer of water-saturated iso-butanol (to ensure formation of an even interface and exclusion of oxygen).

Note: Alternatively, use water or ethanol diluted 1:1 with 0,375 M Tris, pH 8.8.

iv. Allow the gel to set for at least 45 min or until the interface between the gel and the overlay is visible.

v. Pour the liquid from the top of the resolving gel, wash the top of the gel thrice with distilled water, and remove excess liquid by inserting a piece of filter paper between the glass plates and allowing the excess liquid to absorb into filter paper

vi. Place the gel apparatus upright, prepare the 4% stacking gel, and load it on top of the resolving gel. Position the comb immediately.

vii. Allow the gel to polymerize for at least 45 min-1 h before loading the samples.

viii. Remove the comb, and assemble the glass plates in the electrophoresis apparatus.

ix. Add running buffer to the bottom reservoir, and insert the glass plates into the electrophoresis tank. Fill the wells with the electrophoresis buffer, and remove air from under the gel bottom.

x. Mix the rotavirus dsRNA sample (500ng-1µg) with 2X gel loading dye (Appendix)

xi. Load each sample in PAGE buffer into the designated gel wells.

xii. Carryout electrophoresis at electrophoreses at 100 V for 16-20 hr or till the dye front comes out of the gel bottom.

Note: Take all measures while handling acrylamide (a potent neurotoxin). Use protective clothing while handling solutions of acrylamide.

3. Silver staining

i. Pour out the running buffer, and remove the gel from between the glass plates.

ii. Cut the bottom right corner for gel orientation. Discard the stacking gel.

iii. Add 200 ml of fixing solution 1 to each gel, and rotate at room temperature for 30 min on an orbital shaker.

iv. Aspirate fixing solution 1 and replace with 200 ml of fixing solution 2. Rotate for 30 min at room temperature on the orbital shaker.

v. Make up $AgNO_3$ just before use. Work carefully as $AgNO_3$ stains hands and surfaces. Aspirate fixing solution 2, and add 200 ml of silver nitrate staining solution. Rotate for 30 min at room temperature on the orbital shaker.

vi. Prepare developing solution by adding the NaOH to the previously prepared formaldehyde and water solution.

vii. Aspirate the silver nitrate staining solution, and wash the gel twice with distilled water for 2 min each time.

viii. Add approximately 50 ml of developing solution to the gel, and agitate by hand for 30 sec to remove any black precipitate.

ix. Aspirate the developing solution, and then add the remaining developing solution (~200 ml). Rotate for ~5 min at room temperature or until RNA bands are visible.

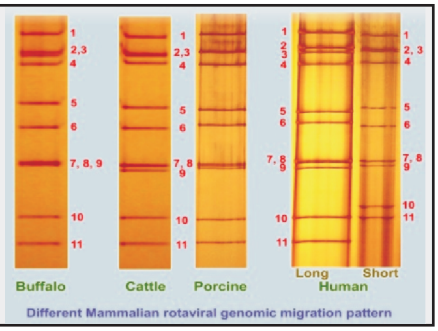
x. Drain off the developing solution, and add the stopping solution to prevent further color development.

xi. Rotate for 5-10 min at room temperature before rinsing the gel in distilled water.

xii. Dry the gel in a standard vacuum gel dryer. The gel can also be temporarily stored in a 20% ethanol/1% glycerol mixture or in a 5% acetic acid solution.

Reading of results

The discerned genome segments will appear brown after silver impregnation as shown below for rotavirus. Different patterns within rotavirus exhibit circulation of variants or electropherotypes.



Different Mammalian rotaviral genomic migration pattern