TRAINING SCHOOL

ONWARD ratHEV

LABORATORY PROTOCOL SERIES

PROTOCOL 1

Dot blot assay

Serology

COST Action CA24140

Hosted by the Clinical Virology and Zoonoses group, IMIBIC

Córdoba, Spain | 9–11 September 2026

Dot blot assay

The aim is to detect the presence of a specific immune response to HEV (P. balayani) and/or ratHEV (R. ratti) in human serum samples. To this end, proteins (elution fractions) from each virus are spotted onto a membrane and incubated with human serum (primary antibody), followed by incubation with an anti-human IgG secondary antibody and chemiluminescent detection. Protein samples are normalized to the same concentration and spotted onto an activated membrane. Following a blocking step, immunodetection (primary and secondary antibodies) is performed, and the signal is visualized by chemiluminescence using an imaging system (Dremsek et al., *Medical Microbiology and Immunology*, 2012; Casares Jimenez et al., *Emerging Microbes & Infections*, 2024).

The ratHEV recombinant protein comprises amino acid residues 315–599 of the capsid protein of the ratHEV strain with GenBank accession number GQ504009, whereas the HEV recombinant protein corresponds to the entire ORF2 and truncated 5′ and 3′ segments thereof, derived from the HEV genotype 3i strain with GenBank accession number FJ705359. As a negative control (C−), a nucleocapsid protein derivative of Puumala orthohantavirus strain Vranica/Hällnäs was used. As a positive control (C+), an *E. coli* M15 lysate was used.

- ## Reagents/Equipment

1. **Blot membrane** (strips) (Ref 88518, ThermoFisher Scientific)
2. **Shaker**
3. **Buckets**
4. **Chemiluminescence Imaging System** (e.g.: ChemiDoc Biorad)
5. **Parafilm**

6. **Damp sponge and filter paper** (Whatman or another brand)
7. **Methanol 100%**
8. **Distilled water**
9. **NaCl 5 M (1 L)**
    a. Dissolve 292.2 g of NaCl in distilled water.
    b. Make up to 1 L.
10. **Tris-HCl 1 M pH 7.5 (1 L)**
    a. Dissolve 121 g of Tris in distilled water.
    b. Adjust the pH to 7.5 with HCl.
    c. Make up to 1 L.
11. **Tris-Buffered Saline with Tween-20** (TBS-T, 1L).
    a. 20 mL Tris-HCl 1 M pH 7,5
    b. 30 mL NaCl 5 M
    c. 1 mL Tween-20
    d. Add distilled water to bring the volume to 1 L
12. **Blocking buffer**: 5% of milk for laboratory use in TBS-T (2.5 g of powdered milk for laboratory use) in 50 mL of TBS-T.:
13. **Secondary Antibody**: goat anti-IgG human (Ref 32935S, Cell Signaling Technology)
14. **Substrate solution**: Clarity Western ECL Kit (ref 1705062, Biorad)

- **<u>Previous steps</u>**
    1. **Protein normalization**
        a. Quantify the proteins of the elution fractions using the Bradford method.
        b. Select elutions with higher concentrations (these are usually elution 3 to 6 among the 10 elutions).

c. Dilute the selected elutions to a final concentration of 0.2 µg/mL (same concentration for all drops).

2. **Preparation of membrane strips**

a. Cut membrane strips 1.5 cm wide × 5 cm long. Label each strip (using a pencil or marker suitable for membranes).

- **Protocol**

1. **Membrane Activation and Hydration**
   a. Activate the membrane by immersing it with 100% methanol for 2 minutes.
   b. Wash with distilled water for 5 minutes
2. **Setup for Protein Deposition**
   a. Place the membrane on a thoroughly moistened sponge.
   b. If the membrane does not lie flat, use moistened filter paper (Whatman).
   c. Roll a Falcon tube (or similar) over the membrane to remove bubbles and ensure even contact.
3. **Sample Deposition (Spots, divide the membrane in four consecutive areas)**
   a. Deposit 5 µL of each protein (814 and 818 at 0.2 µg/mL).
   b. Deposit 5 µL of the negative control (Hantavirus).
   c. Deposit 3 µL of the positive control (C+) E. coli.
   d. Verify that the droplets are well-defined in each well and do not mix.
   e. Allow to dry for approximately 10 minutes.
4. **Post-deposition washing**
   a. Wash the dried membrane by immersing with distilled water for 20 minutes while stirring.
5. **Blocking**

a. Remove washing solution and add 5 mL of blocking buffer (5% powdered milk in TBS-T) and incubate for 1 hour with agitation at room temperature

6. **Washing**
    a. Wash with TBS-T for 5 minutes with agitation.
7. **Incubation with Primary Antibody (serum)**
    a. Prepare the primary antibody (patient serum) at a dilution of 1:2000 using 5 mL of milk/TBS-T + 2 µL of serum.
    b. Incubate overnight (O/N) at 4 °C with gentle agitation.
8. **Washing**
    a. Perform 3 washes with TBS-T, 5 minutes each, with agitation.
    b. Perform 1 additional wash with milk/blocking solution for 5 minutes.
9. **Incubation with secondary antibody**
    a. Prepare the goat anti-human IgG secondary antibody at a 1:8000 dilution using 5% milk/TBS-T + 0.6 µL of secondary antibody.
    b. Incubate for 1 hour at room temperature with gentle agitation.
10. **Washing**
    a. Perform 3 washes with TBS-T, 5 minutes each, with gentle agitation.
    b. Leave the strips in TBS-T of the last wash for the following steps to avoid drying just until the reveal process
11. **Revealing and image acquisition**
    a. Prepare the substrate – Clarity Western ECL Kit – Add the following to a 15-mL Falcon tube:2
        i. 2.5 mL of Luminol/Enhancer Solution
        ii. 2.5 mL of Peroxide Solution
    b. b. Place the strip in a tray and add the substrate until the strip is completely covered. Then, place the strip on a sheet of Parafilm and

transfer it to the ChemiDoc. Processing batches of five strips is recommended to prevent them from drying out.

c. For Chemidoc, use the black tray and program Blots – Chemiluminescence, 3×3 exposure; resolution set to level 2 or 3.

d. Capture images with exposure times ranging from 1 s to 10–30 s.

- **Scheme positions**

  This can be a recommended points order.

Sample	Identification
814	HEV
818	ratHEV
H	Hantavirus (C-)
E.C	E. coli (C+)

- **Interpretation**

To conclude that a patient has been specifically exposed to HEV or ratHEV by dot blot, a dot with equal or higher intensity than the positive control must be observed in the respective HEV or ratHEV squares (see the figure below). If dots are observed in both the HEV and ratHEV squares with an intensity greater than the positive control, the sample should be classified as indeterminate. Due to potential cross-reactivity, it remains unclear whether the patient has been exposed to HEV, ratHEV, or both.

