

Titration of Human Coronaviruses, HCoV-229E and HCoV-OC43, by an Indirect Immunoperoxidase Assay

Francine Lambert, Hélène Jacomy, Gabriel Marceau, and Pierre J. Talbot

Abstract

Calculation of infectious viral titers represents a basic and essential experimental approach for virologists. Classical plaque assays cannot be used for viruses that do not cause significant cytopathic effects, which is the case for strains 229E and OC43 of human coronavirus (HCoV). An alternative indirect immunoperoxidase assay (IPA) is herein described for the detection and titration of these viruses. Susceptible cells are inoculated with serial logarithmic dilutions of samples in a 96-well plate. After viral growth, viral detection by IPA yields the infectious virus titer, expressed as “Tissue Culture Infectious Dose” (TCID₅₀). This represents the dilution of a virus-containing sample at which half of a series of laboratory wells contain replicating virus. This technique is a reliable method for the titration of HCoV in biological samples (cells, tissues, or fluids).

Key words: human coronavirus; HCoV-229E; HCoV-OC43; cell and tissue samples; titration; immunoperoxidase assay; TCID₅₀

1. Introduction

HCoV were first isolated in the mid-1960s from patients with upper respiratory tract disease (1–3). As described by McIntosh (4), the identification of coronaviruses in clinical samples was a very difficult task until the 1970s, as HCoV induced subtle or nonexistent cytopathic effects, and many cells types were not susceptible to the virus. Commonly, HCoV-OC43 (ATCC: VR-759)

was grown and maintained on BS-C-1 (ATCC: CCL-26), RD (ATCC: CCL-136) or HRT-18 (also called HCT-8; ATCC: CCL-224) cell lines. On the other hand, HCoV-229E (ATCC: VR-740) was grown and maintained on WI-38 (ATCC: CCL-75), MRC-5 (ATCC: CCL-171) or L-132 (ATCC: CCL-5) cell lines. Interestingly, cells from the central nervous system (CNS) were reported to be highly susceptible to HCoV replication. The SK-N-SH (ATCC: HTB-11) neuroblastoma and H4 (ATCC: HTB-148) neuroglioma cell lines were highly susceptible to infection, as well as astrocytoma cell lines U-87 MG (ATCC: HTB-14), U-373 MG, and GL-15 (5–8). Even though it was reported that HCoV-229E infectious titers could be determined by plaque assay on specific cell lines, such as MRC-5 or L-132 (6), this proved not to be a reliable assay and, as for HCoV-OC43, an alternative assay was required: an indirect immunoperoxidase assay (IPA) on coronavirus-susceptible cells to quantify infectious virus in biological samples.

The immunoperoxidase assay is an enzymatic antigen detection technique that uses the enzyme horseradish peroxidase (HRP) to label antigen-antibody complexes. The principle of the IPA technique is that a specific antibody recognizes and binds to its specific antigen to yield an antibody-antigen complex. For detection of these complexes, the primary antibody is either directly labeled with HRP or remains unlabeled, with detection achieved by a labeled secondary antibody. If a secondary antibody is used, it must be generated against the immunoglobulins of the animal species in which the primary antibody was produced. The enzyme substrate, 3,3'-diaminobenzidine (DAB) is then converted by HRP to a precipitating brown end-product.

2. Materials

2.1. Preparation of Tissue Samples

1. Digital scales.
2. Ethanol 70% (v/v).
3. Sterile dissection kit.
4. Ice.
5. Sterile PBS.
6. Centrifuge.
7. Sterile tubes: 15 ml for big pieces of tissue or 5 ml for small pieces.

2.2. Preparation of Cell Samples

1. Sterile PBS.
2. Centrifuge.
3. Sterile tubes.
4. Cell medium.

2.3. Preparation of Susceptible Cells

1. L-132 cell line (human lung epithelium; ATCC: CCL5) for HCoV-229E (*see Note 1*).
2. HRT-18 cell line (human adenocarcinoma rectal (**9**)) for HCoV-OC43 (*see Note 1*).
3. Alpha-MEM (alpha minimum essential medium; Invitrogen).
4. Fetal bovine serum (FBS, Hyclone).
5. Trypsin at 0.25% (w/v) for the L-132 cell line or trypsin/EDTA at 0.05% (w/v) for the HRT-18 cell line.
6. 96-well plate flat bottom for tissue culture.
7. Multichannel pipette and sterile tips.
8. Incubator 37°C with 5% (v/v) CO₂.

2.4. Immunoperoxidase Assay

2.4.1. Infection of Cells with Samples to Test

1. Alpha MEM with 1% (v/v) FBS.
2. Sterile paper towels.
3. Sterile tips.
4. Incubator 33°C with 5% (v/v) CO₂.

2.4.2. Virus Detection

1. 100% methanol.
2. Hydrogen peroxide 30% (Sigma).
3. PBS.
4. Primary antibodies (*see Note 2*):
 - a. HCoV-229E: An ascites fluid or culture supernatant from mouse MA b 5-11H.6 (**7, 10**), directed against the surface glycoprotein (S) of HCoV-229E, or equivalent polyclonal antiserum
 - b. HCoV-OC43: An ascites fluid or culture supernatant from mouse MA b 1-10C.3 (**8**), directed against the surface glycoprotein (S) of HCoV-OC43, or equivalent polyclonal antiserum.
5. Secondary antibody: anti-mouse immunoglobulins conjugated to horseradish peroxidase (HRP; from KPL) at 1/500 dilution in PBS.
6. Multichannel pipette and tips.
7. Developing solution: 25–50 µg/100 ml of freshly made DAB (3,3'-diaminobenzidine tetrahydrochloride) solution in PBS. Filter this solution on Whatman paper then add 0.01% hydrogen peroxide (33 µl of H₂O₂ 30%). This solution is light sensitive and toxic.
8. Nonsterile paper tissue.

9. Light microscope.
10. Incubator 37°C.

3. Methods

3.1. Preparation of Tissue Samples

1. Prepare sterile tubes for each organ and determine the weight of the empty tubes.
2. Quickly dissect the organs of interest in sterile conditions and keep them on ice. Rinse instruments in 70% (v/v) ethanol between each dissection.
3. Determine the weight of each biological sample.
4. Homogenize tissue samples to 10% (w/v) sterile PBS with a Polytron homogenizer in a laminar flow hood.
5. Centrifuge tubes at 4°C, for 20 min at 1000 × g.
6. Collect supernatants in new sterile tubes and process to immunoperoxidase detection, or immediately freeze at -80°C and store until assayed.

3.2. Preparation of Cell Samples

3.2.1. Adherent Cells

1. To determine extracellular viral production, collect supernatants in sterile tubes from infected cells (to be tested) at appropriate times following infection. For estimation of intracellular viral titers, remove medium, wash cells in warm (37°C) sterile PBS, then add the same volume or a known volume of medium and perform three cycles of freeze/thaw at -80°C to lyse cells and release viral particles in the medium.
2. Collect medium samples in sterile tubes, centrifuge for 5 min at 1000 × g.
3. Collect supernatants in new sterile tubes and process to IPA detection, or immediately freeze at -80°C and store until assayed.

3.2.2. Nonadherent Cells

1. Centrifuge nonadherent cells at 1000 × g for 5 min.
2. Collect supernatants in sterile tubes; they represent the extracellular portion of infectious virus.
3. Resuspend the cell pellet in the same volume (corresponding to the supernatant) or in a known volume of culture medium.
4. Perform three cycles of freeze/thaw at -80°C to lyse cells and release the viral particles in the medium; they represent the intracellular portion of the infectious virus.
5. Centrifuge for 5 min at 1000 × g.
6. Collect supernatants in new sterile tubes and process to IPA detection, or immediately freeze at -80°C and store until assayed.

3.3. Preparation of Susceptible Cells

To verify the productivity of HCoV infection in biological samples, an immunoperoxidase assay is performed on coronavirus-susceptible cells. Suspensions of susceptible cells are prepared from confluent monolayers by trypsin treatment (trypsin [0.25% (w/v)] for the L132 cell line or trypsin/EDTA [0.05% (w/v)] for the HRT-18 cell line). Trypsin is then inactivated by the addition of culture medium supplemented by 10% (v/v) with fetal bovine serum (FBS). Susceptible cells are plated in a 96-well plate for tissue culture using a multichannel pipette. Dispense 100 μ l/well of cell suspension as follows.

- HCoV-229E: L-132 cells are seeded at 70,000 cells/ml if inoculation of samples is to be performed 2 days later or 50,000 cells/ml if inoculation of samples is to be performed 3 days later, in alpha-MEM supplemented with 10% (v/v) FBS. Incubate at 37°C in a humid atmosphere containing 5% (v/v) CO₂.
- HCoV-OC43: HRT-18 cells are plated at 70,000 cells/ml if inoculation of samples is to be performed 3 days later or 50,000 cells/ml if inoculation of samples is to be performed 4 days later, in alpha-MEM supplemented with 10% (v/v) FBS. Incubate at 37°C in a humid atmosphere containing 5% (v/v) CO₂.

3.4. Immunoperoxidase Assay

3.4.1. Infection of Susceptible Cells with Samples to Be Tested

1. When susceptible cells (L-132 for HCoV-229E or HRT-18 for HCoV-OC43) are at 70–80% confluence in the laminar flow hood, flick the medium of the 96-well plate. Remove residual medium by wrapping each plate in a sterile paper tissue and gently flicking it face down onto sterile paper.
2. Add 100 μ l of alpha-MEM supplemented with 1% (v/v) FBS to each well.
3. Each sample should be tested in four adjacent columns in the same plate. Put 100 μ l/well of aliquots to be tested in the first row.
4. Inoculate with serial logarithmic dilutions of infected samples: Add 11 μ l/well of the same sample again in four wells in the second row of the 96-well plate. Mix with a multichannel pipette by pipetting up and down in the pipette tip three times and transfer 11 μ l/well in the third row and so forth to the last row of the plate (**Fig. 1**). Change tips after every dilution and discard the remaining 11 μ l.
5. Include a positive control in each experiment by inoculating four wells of reference virus.
6. Incubate in a humidified chamber at 33°C with 5% (v/v) CO₂.
 - a. HCoV-229E: 5 days
 - b. HCoV-OC43: 4 days

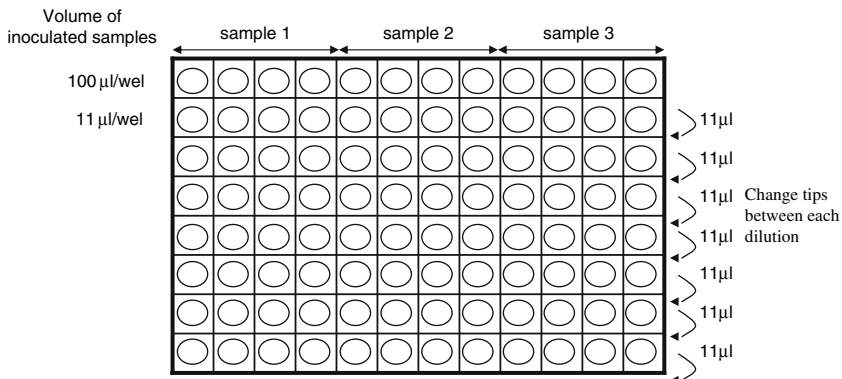


Fig. 1. Schematic representation of a 96-well plate illustrating inoculation and serial logarithmic dilutions of infected samples for titration.

3.4.2. Virus Detection

1. Remove medium completely by flicking the plates onto nonsterile paper towels in the laminar flow hood and delicately rinse the cells by gently filling the wells with PBS with a multichannel pipette. Flick the PBS and remove residual PBS by wrapping each plate in a paper tissue.
2. Fix cells with 100% methanol containing 0.3% (v/v) hydrogen peroxide for 15–30 min at room temperature.
3. Remove fixative by flicking the plates over a sink and remove the residual liquid by flicking it face down onto several paper towels lying on the bench top and then let it totally air-dry, face up for approximately 15–30 min.
4. Prepare an antibody solution specific to the virus at the appropriate dilution in PBS. Add 100 μl of specific viral-antibody to each well and incubate plates for 2 h at 37°C.
5. Completely remove the medium by flicking it over a sink. Rinse the plate by filling the wells with PBS. Flick the PBS into the sink and rinse with PBS twice more, flicking the PBS into the sink after each rinse. Remove residual PBS by flicking it face down onto nonsterile paper towels lying on the bench top.
6. Add 100 μl to each well of secondary antibody at 1/1000 dilution in PBS.
7. Incubate plate for 2 h at 37°C without CO₂ (see **Note 3**).
8. 30 min before the end of incubation, prepare a solution of DAB (detection reagent).
9. Rinse the plate three times in PBS as in step 5.
10. Add 100 μl of developing reagent, DAB solution in PBS, with 0.01% (v/v) hydrogen peroxide to each well and incubate 10–20 min at room temperature, or until the positive control is stained.

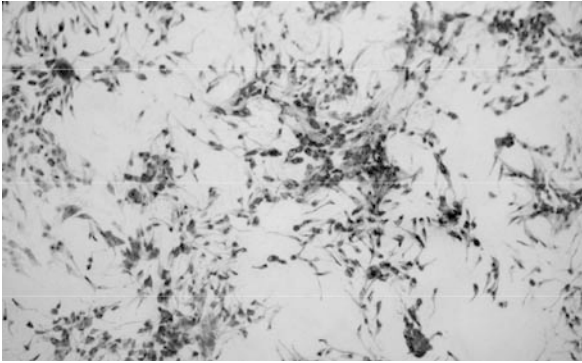


Fig. 2. Example of HRT-18 cells positive for HCoV-OC43. Monolayer of HRT-18 cells were inoculated with an infected biological sample for titration. Four days postinoculation, an indirect immunoperoxidase assay was performed and cells positive for viral antigens appear brown after DAB detection.

11. Stop the reaction with one wash with water as in step 5 and fill each well with 100 μ l deionized water.
12. Read plates with a light microscope to quantify all wells presenting stained cell (**Fig. 2**)

3.4.3. Determination of Titers

Infectious virus titers (tissue culture infectious dose at 50%, TCID₅₀) are calculated by the Karber method (**II**):

$$\text{DICT}_{50} = D - [d(S - 0.5)]$$

where $D = -\log_{10}$ of the last dilution showing 100% (4 wells/4 wells) of virus positive wells; $d = -\log_{10}$ of dilution factor. Example: $-\log_{10} 10 = -1.0$; $S =$ number of all wells presenting virus, including those showing 100% of viral positive wells; this last one representing the unit and other dilutions being a fraction of this unit. For an example see **Fig. 3** (also see **Note 4**).

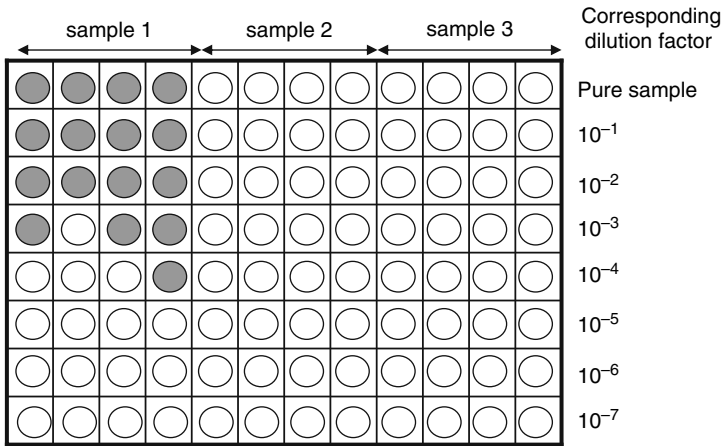


Fig. 3. Schematic representation of a 96-well plate after the immunoperoxidase assay: viral antigen positive wells (as soon as at least two cells are stained brown) are illustrated in gray for the first sample.

Variables:

- 10⁻¹ = 4/4 positive wells = 1.0
- 10⁻² = 4/4 positive wells = 1.0 (*) (**)
- 10⁻³ = 3/4 positive wells = 0.75 (**)
- 10⁻⁴ = 1/4 positive wells = 0.25 (**)
- 10⁻⁵ = 0/4 positive wells = 0.0

(*) used for *D* and (**) used for *S*

Calculation:

$$\begin{aligned}
 \text{DICT}_{50} &= D - [d(S - 0.5)] \\
 &= (*) - [d(**) - 0.5)] \\
 &= 2 - [-1((1 + 0.75 + 0.25) - 0.5)] \\
 &= 2 - [-1(2 - 0.5)] \\
 &= 2 - [-1(1.5)] \\
 &= 2 - [-1.5] \\
 &= 2 + 1.5 \\
 &= 3.5 \log_{10} \text{ (in } 100 \mu\text{l as each well contain } 100 \mu\text{l)}
 \end{aligned}$$

The infectious titer of sample 1 is 4.5 log₁₀/ml or 10^{4.5} TCID₅₀ /ml.

For organs or intracellular viral titers, keep in mind the dilution factor used. Normally tissue samples are diluted at 10% (w/v), so virus titers will be multiplied by 10. In example 1, the virus titer would be 10^{5.5} TCID₅₀ /ml.

4. Notes

1. This protocol can be adapted for many other cell culture systems. HCoV-229E could be grown in normal human fetal lung fibroblast cells MRC-5 (ATCC: CCL-171) or Huh7 (12) and HCoV-OC43 in African green monkey kidney cells

- BS-C-1 (ATCC: CCL-26) or human rhabdomyosarcoma cell line RD-151 (ATCC: CCL-136).
2. This protocol can be adapted using other sources of antibodies; e.g.:
 - a. HCoV-229E: Murine monoclonal antibodies specific for the nucleocapsid protein (N) of HCoV-229E (Chemicon International) (**13**): Clone 401-4A.
 - b. HCoV-OC43: Murine monoclonal antibody to HCoV-OC43 (Chemicon international) (**14**): clone 541-8f or 542-7D.
 - c. An ascites fluid from mouse MAb O.4.3 (**15**), directed against the surface glycoprotein (S) of HCoV-OC43.
 - d. An ascites fluid from mouse MAb 4E11.3 directed against the nucleocapsid (N) protein of the serologically related hemagglutinating encephalomyelitis virus of pigs (**10**).
 3. HRP conjugate is sensitive to CO₂ and will lose its activity.
 4. There are Excel spreadsheets to calculate TCID₅₀/ml on the Internet at, e.g., <http://ubik.microbiol.washington.edu/protocols/bl3/tcid50.htm>

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