Assay for Adherence of *Vibrio cholerae* to Eukaryotic Cell Lines

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[Abstract] *Vibrio cholerae* colonizes the intestinal epithelium and secretes cholera toxin (CT), a potent enterotoxin that causes severe fluid loss characteristic of the disease cholera. *V. cholera*e is a non-invasive Gram-negative bacterium that adheres to intestinal cells as well as a variety of different cell types. A protocol for adherence of *V. cholerae* to various cell lines is described. The adhered bacteria can be used to examine expression of genes that are differentially expressed between adhered and unadhered bacteria or other purposes (Dey *et al.,* 2013).

Materials and Reagents

1. Cell lines: INT 407, Hep-2, Hela, HT-29 (available from ATCC and other commercial sources)
2. *Vibrio cholerae* classical biotype strain O395
3. Dulbecco's modified Eagle's medium (DMEM) (Life Technologies, Gibco®)
   a. High glucose (4,500 mg/L) (catalog number: 12800-017)
   b. Low glucose (1,000 mg/L) (catalog number: 31600-034)
   c. No glucose (catalog number: 11966-025)
4. New born calf serum (NCS) (not heat inactivated) (Life Technologies, Gibco®, catalog number: 16010-159)
5. 10x Trypsin-EDTA (Life Technologies, Gibco®, catalog number: 15400)
6. TritonX-100 (Affymetrix, catalog number: T1001)
7. Dimethyl sulfoxide (DMSO) (Sigma-Aldrich, catalog number: C6164)
8. D-(+)-Glucose (Sigma-Aldrich, catalog number: G7021)
9. D-Galactose
10. 10x cell lysis buffer (Cell Signaling Technology, catalog number: 9803)
11. pNPP (5 mg) (Sigma Chemical, catalog number: N-9389)
12. Bacto tryptone (BD, Difco™, catalog number: 211705)
13. Bacto yeast extract (BD, Difco™, catalog number: 212750)
14. Bacto agar (BD, Difco™, catalog number: 214010)
15. $NaHCO_3$ (Merck KGaA)

16. NaCl (Merck KGaA)
17. Na_2HPO_4 (Merck KGaA)
18. KCl (Merck KGaA)
19. KH_2PO_4 (Merck KGaA)
20. 70% ethanol (Merck KGaA)
21. NaOH (Merck KGaA)
22. Personal protective items (apron, gloves, mask *etc.*)
23. Bio-Rad protein assay dye reagent (Bio-Rad Laboratories)
24. Complete DMEM (100 ml) (see Recipes)
25. Incomplete DMEM (100 ml) (see Recipes)
26. No glucose complete DMEM (100ml) (see Recipes)
27. 10x PBS (500 ml, pH 7.4) (see Recipes)
28. 1x PBS (50 ml) (see Recipes)
29. 1x Trypsin-EDTA (10 ml) (see Recipes)
30. 1% Triton X-100 (50 ml) (see Recipes)
31. 0.9% saline solution (see Recipes)
32. LB medium (100 ml) (see Recipes)
33. LB Agar (100 ml) (see Recipes)
34. Alkaline phosphatase assay buffer (10 ml) (see Recipes)

Equipment

1. 25 cm^2 tissue-culture treated flasks (BD Biosciences, Falcon®, catalog number: 353108)
2. Sterile, disposable 15 ml and 50 ml centrifuge tubes
3. Sterile, disposable 1.5 ml microcentrifuge tubes
4. Sterile microtips
5. Sterile cryovials
6. Cell scraper (BD Biosciences, Falcon®)
7. 0.22 µm filter units (Millex-GP) (Millipore, catalog number: SLGP033RS)
8. CO_2 incubator
9. Laminar flow hood
10. Liquid nitrogen storage container
11. Inverted microscope
12. Centrifuge
13. Microcentrifuge
14. Pipetteman
15. UV-Vis Spectrophotometer

Procedure

Note: For handling cell lines and bacterial cultures, aseptic techniques must be used and work should be carried out in a sterile laminar flow hood when practicable.

A. Culturing of cell lines

1. An aliquot of the chosen cell line (approx. 10^6 cells) stored in liquid nitrogen (see Note 1) was used to seed a 25 cm^2 tissue culture flask containing 5 ml complete DMEM. The cultures were grown to 70-80% confluency (approx. 4 x 10^6 cells) at 37 °C in 5% CO_2 humidified atmosphere. INT 407 was grown in low glucose complete DMEM, Hep-2, Hela and undifferentiated HT-29 were grown in high glucose complete DMEM. Differentiated (see Note 2) HT-29 was grown in no glucose complete DMEM at 10% CO_2 humidified atmosphere.
2. The cultures were washed three times with 5 ml 1x phosphate buffered saline (PBS) and 4 ml low glucose incomplete DMEM was added.

B. Bacterial cell culture

1. A loopful of *V. cholerae* strain O395 preserved at -70 °C in LB medium containing 20% glycerol was streaked on LB agar plates and incubated at 37 °C for 16-18 h.
2. A loopful of *V. cholerae* culture from the plate was inoculated into 5 ml LB medium and grown with aeration (shaking at approx. 200 rpm in a rotary shaker) at 37 °C for 16-18 h. From this culture, a 1% inoculum (50 µl) was added to 5 ml LB medium and grown to the mid logarithmic phase (OD_{600} 0.5) with shaking at 37 °C.
3. The *V. cholerae* culture was centrifuged (5,000 *x g*, 5 min), washed with 5 ml 1x PBS, and suspended in low glucose incomplete DMEM at a concentration of 10^9 colony forming units (CFU) per ml (Bacterial cells from approximately 2 ml logarithmic phase cultures in LB medium were suspended in 1 ml incomplete DMEM).

C. Bacterial adherence to cell lines

1. Cell lines grown to 70-80% confluency in 25 cm^2 flasks were used.
2. *V. cholerae* culture (200 µl, approximately 2 x 10^8 CFU) in low glucose incomplete DMEM was added to the 70-80% confluent cell lines, resultant multiplicity of infection (MOI) was about 50.
3. After addition of bacteria, the cell lines were incubated for 45 min at 37 °C in 5% CO_2 humidified atmosphere for optimum adherence.
4. After 45 min, the supernatant was discarded and non-adhered bacteria were removed by washing the infected cell line 4 times with 5 ml 1x PBS and then low glucose complete DMEM (5 ml) was added and incubated for different periods of time (1-12 h).

5. After the desired time period, the supernatant was discarded and the infected cell line was washed 4 to 5 times with 5 ml 1x PBS.
6. 1 ml of 1% Triton X-100 was added and incubated for 2 min at room temperature with gentle swirling when the cell line could be seen to lyse and detach from the flask. The total culture was collected in 1.5 ml micro centrifuge tubes and vigorous pipetting or vortexing was done. The Triton X-100 treatment lysed the eukaryotic cells but had no effect on the viability of the bacterial cells (see Note 3).
7. The bacterial cells were collected by centrifugation (5,000 *x g*, 5 min) at room temperature and the pellet was suspended in 1 ml 1x PBS.
8. From this suspension 10 µl was added in 990 µl of 0.9% saline solution and bacterial CFU was determined by serial dilution and plating on LB agar plates. Remaining adhered bacterial cells could be used for gene expression studies or other purposes.
9. Parallel experiments were performed with *V. cholerae* under identical conditions but without cell line as an unadhered control.

Notes

1. Cell lines were stored in liquid nitrogen with 10% DMSO as a cryoprotectant.
2. HT29 differentiation
   a. Differentiation of the HT29 cells was performed essentially as described by Huet *et al.,* (1987).
      i. Undifferentiated HT29 cells were grown to 70-80% confluency in high glucose complete DMEM in 25 cm^2 flasks.
      ii. 1 ml of a solution containing 0.5% trypsin and 1 mM EDTA was added, after about 5 min the detached cells were used to seed a 25 cm^2 flask containing no glucose complete DMEM supplemented with 20 mM glucose and 1 mM galactose (day 0).
      iii. Then the following schedule was followed to gradually increase the concentration of galactose to 5 mM and decrease the concentration of glucose: (a) day 1, 15 mM glucose plus 1 mM galactose; (b) day 2, 10 mM glucose plus 3 mM galactose; (c) day 3, 5 mM glucose plus 4 mM galactose; (d) day 4, 5 mM galactose. The medium was changed every day over the period of 4 days. Once adapted, cells were grown for several passages in no glucose complete DMEM containing galactose (5 mM).
   b. Alkaline phosphatase (AP) activity was estimated as a marker of HT29 differentiation (Luongo *et al.*, 2002).
      i. Differentiated and undifferentiated HT29 cells were released from the 70-80% confluent flask by treatment with 1 ml trypsin (0.5%) - EDTA (1 mM) solution,

washed three times with 1 ml cold 1x PBS and lysed on ice with 1x cell lysis buffer.

ii. For AP activity assay, 50 µl cell lysate was incubated with 1 ml p-nitrophenyl phosphate (1 mg/ml) in AP assay buffer for 30 min at room temperature and the reaction was stopped by adding 100 µl of 0.1M K_2HPO_4.

iii. AP activity was calculated as absorbance at 405 nm (A_{405}) per mg of total cellular protein. A_{405} was measured against lysates incubated without substrate.

iv. Total protein was estimated by Bradford method using the Bio-Rad protein assay dye reagent.

3. To examine if 1% Triton X-100 treatment had any effect on bacterial cells, a bacterial culture (OD_{600} 0.5 approximately) was divided into 2 equal parts, one part was treated with Triton X-100 for 2 min, and CFU of the treated and untreated culture was determined. If CFU of the two cultures are comparable, it may be assumed that 1% Triton X-100 treatment had no effect on the viability of the bacterial cells used in the study.

Recipes

1. Complete DMEM (100 ml)
   1.0 g DMEM (low or high glucose)
   0.37 g sodium bicarbonate
   10 ml NCS (heat-inactivated for 30 min in a 56 °C water bath)
   Volume adjusted to 100 ml with deionised water
   Filtered using 0.22 µm filter units
   Stored at 4 °C
2. Incomplete DMEM (100 ml)
   1.0 g DMEM (low glucose)
   0.37 g sodium bicarbonate
   Volume adjusted to 100 ml with deionised water
   Filtered using 0.22 µm filter units
   Stored at 4 °C
3. No glucose complete DMEM (100ml)
   89 ml liquid DMEM (no glucose)
   10 ml heat inactivated NCS
   Filtered using 0.22 µm filter units
   Stored at 4 °C
4. 10x PBS (500 ml, pH 7.4)
   40.0 g NaCl

1.0 g KCl

6.07 g Na_2HPO_4

1.0 g KH_2PO_4

Volume adjusted to 500 ml with deionised water, if required pH adjusted with NaOH

Autoclaved

5. 1x PBS (50 ml)

   5 ml of 10x PBS diluted in 45 ml of deionised water

   Filtered using 0.22 µm filter units

6. 1x Trypsin-EDTA (10 ml)

   1 ml of 10x Trypsin-EDTA diluted in 9 ml of sterile 1x PBS

   1 ml aliquots distributed in centrifuge tubes

   Stored at 4 °C

7. 1% Triton X-100 (50 ml)

   0.5 ml 100% Triton X diluted in 49.5 ml of sterile 1x PBS

8. 0.9% saline solution

   0.9 g NaCl in 100 ml deionised water

   Autoclaved

9. LB medium (100 ml)

   1 g Bacto tryptone (1%)

   0.5 g Bacto yeast extract (0.5%)

   0.5 g NaCl (0.5%)

   Volume adjusted to 100 ml with deionised water, pH adjusted to 8.6 with NaOH

   Autoclaved

10. LB Agar (100 ml)

    100 ml LB medium

    1.5 g Bacto agar (1.5%)

    Volume adjusted to 100 ml with deionised water, pH adjusted to 7.4 with NaOH

    Autoclaved

11. Alkaline phosphatase assay buffer (10 ml)

    1 ml 1 M Tris/HCl (pH 9.5) (final concentration 100 mM)

    50 µl 1 M $MgCl_2$ (final concentration 5 mM)

Volume adjusted to 10 ml with deionised water

Autoclaved

Acknowledgments

This protocol is adapted from Huet *et al.* (1987); Luongo *et al.* (2002); and Dey *et al.* (2013).

References

1. Dey, A. K., Bhagat, A. and Chowdhury, R. (2013). Host cell contact induces expression of virulence factors and VieA, a cyclic di-GMP phosphodiesterase, in *Vibrio cholerae*. *J Bacteriol* 195: 2004-2010.
2. Huet, C., Sahuquillo-Merino, C., Coudrier, E. and Louvard, D. (1987). Absorptive and mucus-secreting subclones isolated from a multipotent intestinal cell line (HT-29) provide new models for cell polarity and terminal differentiation. *J Cell Biol* 105: 345-357.
3. Luongo, D., Mazzarella, G., Della, R. F., Maurano, F. and Rossi, M. (2002). Down-regulation of ERK1 and ERK2 activity during differentiation of the intestinal cell line HT-29. *Mol Cell Biochem* 231: 43-50.

