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Assessing *Pseudomonas* Virulence Using Host Cells

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Abstract

While human or animal models are often considered the gold standard experimental system for defining virulence factors, cell culture-based infection models have proven useful for identifying important virulence factors and for examining the interactions between pathogens and the epithelial barrier. The first step in infections for most mucosal pathogens involves binding (adhesion) to the epithelial cells that line the mucosa. Successful pathogens can then penetrate the barrier by (1) inducing their uptake (i.e., “entry” or “invasion”) into epithelial cells, (2) crossing the barrier by inducing epithelial cell death, and/or (3) penetrating between cells. This chapter describes growth conditions to form polarized cultures, either two-dimensional monolayers or three-dimensional cysts, of various immortalized epithelial cell lines. It describes assays to measure key early interactions between *P. aeruginosa* and host cells, including binding, invasion, and cytotoxicity. Many virulence factors defined by these criteria have been shown to be important for pathogenesis of *P. aeruginosa* infections in animals or humans. These methods are also applicable to other pathogens.

Keywords

Pseudomonas aeruginosa; Polarized epithelial cells; Transwells; Adhesion; Invasion; Cytotoxicity; Cysts

1 Introduction

1.1 Cell Culture Models for Polarized Epithelium

The use of polarized epithelial cell culture models to study bacterial host interactions has proven informative and often predictive of bacterial interactions with the mucosal barrier in vivo [1]. The mucosal barrier is lined by one or more layers of cells that have distinct apical and basolateral surfaces, separated by tight junctions, that form selective permeability barriers between biological compartments [5]. The apical surface of the cells faces either the outside world or the lumen of a cavity depending on the type of tissue, while the basolateral surface faces adjoining cells and the basement membrane. The apical and basolateral membrane domains are distinguished by unique assemblies of proteins, lipids, and other macromolecules and structures, such as the secreted mucous layer, and have distinct functions.

While many investigations of host–pathogen interactions are performed on primary or immortalized tissue culture cells grown on conventional tissue culture plastic surfaces, these cells cannot completely polarize, as they are forced to mislocalize basolateral transporters to the apical surface in order to obtain nutrients. However, epithelial cells can be plated on

porous filter supports, such as polycarbonate Transwell filters, so that they can be bathed in media from both the apical and basolateral sides when submerged in media in multiwall plates. Under these conditions, the cells can obtain nutrients from either the basolateral or apical surface and will form polarized monolayers when they reach confluence. Filters are available that have an average pore size of 0.4 μm (through which bacteria cannot pass) or 3.0 μm (through which bacteria can pass).

Several immortalized epithelial cell lines, including Manin-Darby Canine Kidney (MDCK, dog kidney epithelial) cells, Calu-3 cells (a human airway epithelial cell line), and 16HBE14o-cells (a human bronchial epithelial cell line), as well as primary human alveolar type II cells, can be plated at “instant” monolayer densities such that they form intact functional two-dimensional polarized monolayers within 24 h [6-9]. Importantly, these cell lines exhibit robust contact-dependent inhibition and thus do not form multilayers of cells. Another useful property is that the longer the monolayers are cultured, the more differentiated and polarized the monolayers become. We define functional polarized monolayers as cells with (1) functional tight junctions, as evidenced by lack of permeability to small molecules such as FITC-inulin, (2) an elevated transepithelial resistance, and (3) functional adherens junctions, with distinct localization of apical and basolateral markers. For MDCK cells plated at an instant monolayer density and cultured on transwells for 24 h, gp135 (podoxcalyxin) is primarily apical whereas p58 (the β-subunit of Na^+/K^+ ATPase) is primarily basolateral [10]. Basolateral localization of the epidermal growth factor receptor, however, requires longer culture times. By varying the length of time in culture, it is possible to recapitulate epithelial development and to compare “incompletely polarized” versus “fully polarized” epithelial monolayers. However, it is important to emphasize that even in “incompletely polarized” monolayers, a functional barrier is formed, with intact tight junctions and adherens junctions.

Using this system, early studies in our lab revealed a strong correlation between the virulence of various strains of *P. aeruginosa* in animal models of acute pneumonia with their ability to cause necrotic death when applied to the apical surface of Transwell-grown polarized MDCK cells, indicating that polarized cells model important aspects of human disease [7]. Studies in which a transposon mutant library of *P. aeruginosa* was screened for mutants that failed to damage polarized MDCK cells when applied apically were important in the discovery of the role of the *P. aeruginosa* type III secretion system [11], which is required for virulence in a murine pneumonia model [12-14] and whose presence correlates with outcomes in human ventilator-associated pneumonia [15, 16]. In more recent studies, this system has allowed us to compare the binding properties, signalling, and entry properties of *P. aeruginosa* when added to the apical versus basolateral surfaces. Our studies have revealed that the *N*-glycan chains at the apical surface and heparin sulfate chains at the basolateral surface are necessary and sufficient for binding, invasion, and cytotoxicity to kidney and airway cells grown at various states of polarization on Transwell filters. In incompletely polarized epithelium, heparin sulfate proteoglycan abundance is increased at the apical surface, explaining its enhanced susceptibility to *P. aeruginosa* colonization and damage [17]. In subsequent work, we found that binding to the *N*-glycans at the apical

surface is primarily mediated by type IV pili, while binding to the basolaterally localized heparin sulfate proteoglycans is mediated by flagella [8].

Whereas two-dimensional cell culture systems have provided many important insights into bacterial pathogenesis, they are some limitations in the extent to which they model the complexity of an intact three-dimensional tissue. For instance, cell–cell and cell–matrix adhesion, gene expression, and orchestration of signalling pathways differ in the absence of a three-dimensional microenvironment. One practical disadvantage of using a two-dimensional MDCK monolayer is that it is difficult to infect cells from the basolateral surface, because the filter blocks access of bacteria to the cells, although this problem can be partially overcome by using filters with larger 3 μm pore size.

Recently, it has become possible to grow MDCK and some other epithelial cell lines in thick, three-dimensional gels of extracellular matrix (ECM), such as collagen I or Matrigel, a reconstituted basement membrane-like material secreted by the Engelbreth Holm Swarm tumor [18]. When MDCK cells are seeded as single cells in collagen gel, they proliferate and over 7 days form a hollow cyst lined by a monolayer of epithelial cells. The apical surfaces face inward towards a central lumen. Thus, epithelial cells can organize into a rudimentary organ-like structure. This process resembles the branching morphogenesis found during formation of many epithelial organs. These organotypic three-dimensional cultures much more closely mimic the normal environment and allow cells to respond more physiologically. Inside–out cysts, with reversed polarity, can be formed by inhibiting integrin-mediated adhesion to the ECM during cyst growth. Although much of the recent work with three-dimensional cultures has focused on cancer and related issues of differentiation [19], these three-dimensional culture systems are beginning to be applied to the study of host–pathogen interactions [17, 20]. However, due to the avid binding of *P. aeruginosa* to ECM, adhesion only be assessed by immunofluorescence microscopy [17].

1.2 Growth and Culture of P. aeruginosa

Most of the assays described below are carried out with commonly used well-characterized strains (PAK, PAO1, PA103) which were originally isolated from human infections but have since been passaged in the laboratory, but in theory any strain could be used. PAK expresses the major adhesins, type IV pili and flagella, and encodes and translocates into the host cell the type III secreted toxins ExoS, ExoT, and ExoY. PAO1, the first *P. aeruginosa* strain to be sequenced, also expresses type IV pili and flagella, as well as ExoS and ExoT. However, compared to PAK, lower amounts of type III secreted effectors are produced. PAO1 exhibits less cytotoxicity towards epithelial cells in culture and is less virulent in a murine model of acute pneumonia compared to PAK [7]. PA103, expresses type IV pili but not flagella, encodes and produces ExoU, a potent phospholipase, and lacks the ExoS gene [12, 21, 22]. When cocultivated with culture epithelial cells, PA103 is highly cytotoxic due to ExoU. Of the three strains, it is the most virulent in an animal pneumonia model [7].

1.3 Adhesion and Invasion Assays

The first step in establishing *P. aeruginosa* infection is receptor-mediated binding to the epithelium on the apical and/or basolateral surface, leading to bacterial internalization and/or

direct host injury as well as dissemination to distant tissues and organs (reviewed in ref. [23]). *P. aeruginosa* encodes multiple adhesins that may account for its ability to bind to the multitude of host cell types that it may encounter in diverse environments, ranging from single-celled amoeba to human epithelial cells. The most important adhesins are Type IV pili, polar fimbriae that undergo extension and retraction, and flagella, which are polar organelles that also mediate swimming motility. Other minor adhesins have also been identified, including the cup fimbriae and the lectin-like proteins, LecA and LecB. A variety of host cell receptors have been identified. These include asialoGM1, mannose-containing *N*-glycoproteins, integrins, heparin sulfate proteoglycans, and the cystic fibrosis membrane receptor (reviewed in ref. [1]).

In cell culture-based models, bacterial adhesion can be monitored visually by microscopy, but quantitation is difficult and time-consuming [24]. Alternatively, adhesion can be measured by releasing surface-bound bacteria after gentle lysis of host cells under conditions that do not cause bacterial lysis (0.25 % Triton-X 100) [25]. *P. aeruginosa* binding to host cells can be measured reasonably reliably and robustly using this latter method [26]. However, given that *P. aeruginosa* also binds avidly to abiotic surfaces such as plastic, care must be taken to wash the epithelial cell monolayer thoroughly. Performing adhesion assays on confluent transwell grown cells offers several additional advantages. First, the filter can be excised and washed thoroughly, so that only binding to host cells (and not to surrounding plastic surfaces) is measured. Second, if the epithelial cells are grown as confluent monolayers on filters with 3 μm pores, bacteria can be added from either the apical or the basolateral side, allowing comparisons [8, 17, 27]. Third, since epithelial cell polarity can be controlled or perturbed when grown on porous filters, binding, invasion, or cytotoxicity as a function of cell polarity can be investigated [8, 17, 27].

In addition to assays that utilize microscopy, a significant advance in quantitating bacterial entry or invasion into cells was the development of the aminoglycoside exclusion assay [25]. This assay is similar to the adhesion assay, except that following the adhesion step (typically 1 h), an aminoglycoside antibiotic, which does not accumulate intracellularly, is added to kill extracellular bacteria for 1–2 h. Internalized bacteria are released by gentle host cell lysis. There are two important caveats to the interpretation of this assay. First, although it is possible that extracellular adherent bacteria may be protected from antibiotics, this has rarely been reported. Second, if the host cell is permeabilized during the incubation period, then aminoglycoside antibiotics may accumulate intracellularly and kill intracellular bacteria. For these reasons, it is helpful in initial experiments to perform cytotoxicity assays in parallel with the adhesion and/or invasions assays to ensure that the integrity of the host cell plasma membrane has not been affected.

Although *P. aeruginosa* is primarily considered an extracellular pathogen, many clinical and laboratory isolates demonstrate measurable internalization by various assays (reviewed in ref. [23]). Importantly, different strains and mutants vary in their internalization efficiency into cultured cells, but all strains are capable of entering into both phagocytic and non-phagocytic cells to some degree [28]. The observation that the ability to enter cells has been maintained during the evolution of *P. aeruginosa* indicates that it is likely to play a fundamental role in the pathogenesis of *P. aeruginosa* infections and/or in surviving in the

environment. Both genetic and cell biologic approaches have revealed that *P. aeruginosa* internalization requires rearrangement of the actin cytoskeleton through pathways involving Abl kinase, the adaptor protein Crk, the small GTPases Rac1 and Cdc42, and p21-activated kinase [29]. The type III secreted effectors ExoS and ExoT target this pathway by interfering with GTPase function and, in the case of ExoT, by abrogating *P. aeruginosa*-induced Abl-dependent Crk phosphorylation [29]. Quantitative adhesion and invasion assays may serve as useful endpoints/readouts to assess potential inhibitors of *P. aeruginosa* infection. In addition, understanding the mechanism of bacterial entry into host cells may reveal new insights into eukaryotic cell biology.

1.4 Cytotoxicity Assays

Bacteria can induce many types of cell death in eukaryotic host cells, including necrosis, apoptosis, and pyroptosis (reviewed in ref. [30]), but most types of cell death ultimately result in loss of membrane integrity and release of cytoplasmic content (cytotoxicity). While there are many methods for quantifying cytotoxicity, commercially available colorometric non-radioactive assays that quantify the release of the stable cytoplasmic enzyme lactate dehydrogenase (LDH) have proven very robust and sensitive. A genome-wide transposon mutant screen of PA103 in which mutants were screened for loss of cytotoxicity when applied to the apical surface of polarized MDCK cells led to the identification of type III secreted effector ExoU [26]. ExoU producing strains are highly virulent in a murine model of acute pneumonia and are associated with worse clinical outcomes in human infections [12,15, 31, 32].

2 Materials

2.1 Cell Culture

1. MDCK clone II cells (ATCC CCL-34**)** (*see* **Note 1**), Calu-3 cells (ATCC HTB-55), and 16HBE14o- and CFBE14o-cells (obtained from Dr. Alan Verkman, UCSF).
2. Modified Eagle Media (MEM) with Earl’s Balanced Salt Solution.
3. Trypsin.
4. Glutamine.
5. Fetal bovine serum (FBS; Thermo Scientific; product code SH30910.03).
6. 10 cm (Corning product code 430293) and 6-well plastic cell (Falcon product code 353046) culture plates.
7. 0.4 μm pore size 12 mm.
8. 3 μm pore size 12 mm Transwell filters (Corning Costar; product code 3402).
9. 8-well Lab-Tek Coverglass chambers (Nalge Nunc International; product code 155409).
10. 100 % Matrigel (BD Biosciences; product code 356234). Store at −20 °C.
11. Anti-β_1 integrin (AIIB2; available from Caroline Damsky through Developmental Studies Hybridoma Bank).

12. Type I collagen solution: 66 % (3 mg/ml) PureCol Vitrogen (Advanced BioMatrix; product code 5005-B), 1× MEM, 2.35 mg/ml $NaHCO_3$, and 0.025 M HEPES, pH 7.6. Store components separately at 4 °C. Prepare the solution fresh and keep on ice.

2.2 Bacterial Culture

1. 50-ml Conical tubes (Falcon; product code 352098).
2. Luria Bertani (LB) broth and plates.

2.3 Bacterial Adhesion and Invasion Assays

1. Gentamicin (Fisher Scientific; product code BP918).
2. Amikacin (Fisher Scientific; product code BP2643).

2.4 Cytotoxicity Assays

1. CytoTox 96® Non-Radioactive Cytotoxicity Assay (Promega; product code G1780).
2. Plate reader or spectrophotometer.

3 Methods

3.1 Culture of Epithelial Cells as Polarized Monolayers on Transwell Filter Supports

3.1.1 MDCK Cells

1. Grow MDCK cells in MEM supplemented with 5–10 % FBS at 37 °C with 5 % CO_2 in 10 cm plastic cell culture plates. When cells reach 100 % confluence, remove cells for splitting by adding 0.25 % Trypsin without EDTA and incubate for 30–40 min at 37 °C. After the cells come off the plastic, pellet them by centrifugation for 15 min at 220 × G, resuspend in MEM + FBS, and passage by diluting them 1:4–1:6. We routinely omit antibiotics for cell culture as they can inhibit the growth of *P. aeruginosa*.
2. For infection with bacteria on the apical surface, the MDCK cells should be grown on either 0.4 or 3 μm filters. For infections with bacteria on the basolateral surface, MDCK cells must be grown on 3 μm filters. If apical versus basolateral infection will be compared, grow the MDCK cells on 3 μm filters.
3. To grow incompletely polarized monolayers, add 0.5–0.7 × 10^6 cells (as determined by counting in a hemacytometer) in 0.5 ml MEM with 5–10 % FBS in the upper chamber of 12-mm Transwell filters, add 1.5 ml MEM with 5–10 % FBS to the lower chamber, and culture cells for 1–3 days at 37 °C with 5 % CO_2. Confluent monolayers plated on 12 mm Transwell filters contain ~2 × 10^6 cells.
4. To grow as polarized monolayers, add 0.4–0.6 × 10^6 cells in 0.5 ml MEM with 5–10 % FBS in the upper chamber of 12-mm Transwell filters placed in the well of a 6-well dish, add 1.5 ml MEM with 5–10 % FBS to the lower chamber, and culture

cells for 5 days at 37 °C with 5 % CO_2. Confluent monolayers plated on 12 mm Transwell filters contain ~2 × 10^6 cells.

3.1.2 Calu-3 Cells: Human Airway Epithelial Cells

1. Grow Calu-3 cells in MEM supplemented with 10 % FBS and 2 mM L-glutamine at 37 °C with 5 % CO_2 in plastic cell culture plates. To split cells for passaging, add 0.25 % Trypsin without EDTA for 40–60 min at 37 °C, dilute into media, passage by diluting them 1:2–1:4 (*see* **Note 2**).
2. To grow polarized monolayers, add 1 × 10^6 cells in 0.5 ml MEM with 10 % FBS and 2 mM L-glutamine in the upper chamber of 12-mm Transwell filters, add 1.5 ml MEM with 10 % FBS and 2 mM L-glutamine to the lower chamber, and culture cells for 3–5 days (incompletely polarized) or 9–12 days (more fully polarized) at 37 °C with 5 % CO_2. Wash once and change media every day. For the last 2 days, grow cells in an air–liquid phase by removing media from the upper chamber of the Transwell filter.

3.1.3 16hbe14o- and cfbe14o-Cells: Human Bronchial Epithelial Cells

1. Grow cells in MEM supplemented with 10 % FBS and 2 mM L-glutamine at 37 °C with 5 % CO_2 in plastic cell culture plates. To split cells for passaging, add 0.25 % Trypsin without EDTA for 40–60 min at 37 °C, centrifuge, resuspend in medium, and dilute 1:4–1:6 (*see* **Note 2**).
2. To grow polarized monolayers, seed 0.5–0.7 × 10^6 cells in 0.5 ml MEM with 10 % FBS and 2 mM L-glutamine in the upper chamber of 12-mm Transwell filters, add 1.5 ml MEM with 10 % FBS and 2 mM L-glutamine to the lower chamber, and culture cells for 2–3 days (incompletely polarized) or 5 days (more fully polarized) at 37 °C with 5 % CO_2. Wash once and change media every day. For the last 2 days, grow cells in an air–liquid phase by removing media from the upper chamber of the Transwell filter.

3.2 Growth of MDCK Cells as Three-Dimensional Cysts

3.2.1 Method A

1. Cover the surface of 8-well Lab-Tek Coverglass chambers with 3 μl of 100 % Matrigel. Remove a portion of 100 % Matrigel from a stock stored at −20 °C. Keep at 4 °C and store on ice during the experiment. Matrigel stored at 4 °C can be used for approximately 3 weeks.
2. Prepare a single cell suspension of 1–3 × 10^4 cells/ml in pre-warmed MEM supplemented with 10 % FBS and 2 % Matrigel.
3. To grow cysts with reversed polarization, add 1:100 dilution of anti-β_1 integrin (AIIB2) to a cell suspension.
4. Add 300 μl of cell suspension to each well of the coverglass chambers and grow for 2–4 days at 37 °C with 5 % CO_2.

3.2.2 Method B

1. Cover the surface of 8-well Lab-Tek Coverglass with 3 μl of 100 % Matrigel.
2. Prepare a single cell suspension of 4×10^4 cells/ml in a type I collagen solution. Keep the solution on ice before addition of cells to avoid solidification of the collagen.
3. To grow cysts with reversed polarization, add 1:100 dilution of anti-β_1 integrin (AIIB2) to a type I collagen solution.
4. Add 250 μl of a cell suspension in type I collagen to each well of coverglass chambers.
5. Allow cells to settle close to the bottom of the well by keeping chambers inside a tissue culture hood for 20 min.
6. Allow the collagen mixture to solidify fully into a gel by putting chambers inside a tissue culture incubator for 20–30 min and then add 200 μl of MEM supplemented with 10 % FBS on top.
7. Change medium every 2 days and grow cysts for 4–7 days.

3.3 Bacterial Culture

1. Inoculate one colony of bacteria from a freshly streaked Luria-Bertani (LB) plate into 5 ml LB broth in a 50-ml conical tube and grow bacteria shaking (300 RPM) overnight (~18 h) at 37 °C (*see* **Note 3**). To maintain aerobic growth conditions during overnight growth, either (1) loosen the cap and tape it to the tube or (2) remove the cap and cover the tube with parafilm.
2. If exponential phase *P. aeruginosa* are to be used, dilute overnight grown (stationary phase) bacteria 1:40 in LB, and grow in 2 ml LB shaking at 37 °C for additional 2–3 h to a final OD_{600} of 0.2–0.6 (*see* **Note 4**).

3.4 Bacterial Infections of Polarized Monolayers

3.4.1 Apical Infections of Two Dimensional Monolayers

1. Prior to addition of bacteria, gently aspirate media from the upper and lower chamber of Transwell filters using a Pasteur pipet connected to a vacuum line. Wash cells once with 0.5 ml (upper chamber) or 1.5 ml of MEM (lower chamber).
2. Dilute log phase or stationary phase bacteria in 200 μl of MEM to achieve the target multiplicity of infection (MOI) based on OD_{600} measurements (*see* **Note 5**). Typical MOIs for adhesion and invasion assays range from 5 to 30. Gently add MEM containing the bacteria inoculum to the upper chamber of the Transwell insert and, without disrupting bacterial suspension, add remaining 300 μl of MEM on top.
3. Incubate at 37 °C with 5 % CO_2 for 1 h.
4. Quantify the exact MOI by plating tenfold serial dilutions of the bacterial culture onto LB plates and incubating overnight to enumerate CFUs.

3.4.2 Basolateral Infection of Two Dimensional Monolayers

1. Prior to addition of bacteria, gently aspirate media from the upper and lower chamber of Transwell filters using a Pasteur pipet connected to a vacuum line. Wash cells once with 0.5 ml (upper chamber) or 1.5 ml of MEM (lower chamber).
2. Dilute log phase or stationary phase bacteria in 50 μl of MEM to achieve the target MOI based on OD_{600} measurements. Typical MOIs for adhesion and invasion assays range from 5 to 30.
3. Place the 50-μl drop in the middle of the well of a 6-well cell culture plate.
4. Gently place the Transwell (on which the polarized cells have been plated) on top of the drop, and cover the plate with a lid.
5. Incubate at 37 °C with 5 % CO_2 for 1 h.
6. Quantify the exact MOI by plating tenfold serial dilutions of the bacterial culture onto LB plates and incubating at 37 °C overnight to enumerate CFUs.

3.4.3 Infections of Cysts

1. For cysts grown by Method I, remove the Matrigel suspension by pipette and wash twice with 300 μl of MEM with a pipette. The majority of cysts will remain at the bottom of the well embedded in 100 % Matrigel.
2. For cysts grown by Method II, using a Pasteur pipet connected to a vacuum line, remove MEM from the top of a type I collagen solution. Add collagenase type VII at 100 U/ml in PBS to each well for 20 min at 37 °C to digest the collagen gel. Remove digested collagen with a pipette, wash twice with MEM by adding and removing 300 μl of MEM with a pipette.
3. Add 300 μl of GFP-expressing bacteria in MEM with 10 % FBS at an MOI of 100–200.
4. Incubate at 37 °C with 5 % CO_2. The length of time for the incubation will depend on whether adhesion, invasion, or cytotoxicity assays will be performed.

3.5 Apical Bacterial Adhesion and Invasion Assays of Two Dimensional Monolayers

1. Gently aspirate media from the sides of the well (to avoid disrupting the monolayers) from the upper and lower chamber using a Pasteur pipet connected to a vacuum line. Wash upper and lower chambers three times gently with 0.5 and 1.5 ml PBS (room temperature), respectively (*see* **Note 6**).
2. For adhesion assays, proceed directly to **step 4**.
3. For invasion assays, add 0.5 and 1.5 ml of MEM containing 0.4 mg/ml amikacin or 0.25 mg/ml gentamicin to the upper and lower chamber, respectively, for 2 h to kill extracellular bacteria (*see* **Note 7**). At then end of incubation with antibiotics, remove media from the upper and lower chamber using a Pasteur pipet connected to a vacuum line, and wash upper and lower chambers twice with 0.5 and 1.5 ml PBS, respectively.

4. In a sterile 50 ml conical tube, add 1 ml of 0.25 % Triton X-100 and three sterile glass beads. Using a sterile #11 scalpel and tweezers, gently excise each filter from the Transwell insert and place in the 50 ml tube. Incubate the filters in the 50 ml tubes at room temperature for 30 min. Vortex each tube every 15 min for 10–15 s to ensure thorough host cell lysis.
5. Prepare tenfold serial dilutions in 1 ml of ul LB of cell lysates. Plate 100 μl onto LB plates and incubate at 37 °C overnight to enumerate CFUs.

3.6 Basolateral Adhesion and Invasion Assays of Two Dimensional Monolayers

1. After basolateral infection of the monolayer, use sterile tweezers to transfer the Transwell filters into a 12-well plate.
2. Remove media from the upper chamber using a Pasteur pipet connected to a vacuum line, and wash upper and lower chambers three times with 0.5 and 1.5 ml PBS (room temperature), respectively.
3. For adhesion assay, proceed directly to **step 5**.
4. For invasion assays, add 0.5 and 1.5 ml of MEM containing 0.4 mg/ml amikacin or 0.25 mg/ml gentamicin to the upper and lower chamber, respectively, for 2 h to kill adherent bacteria (*see* **Note 7**). After incubation with antibiotic, remove media from the upper and lower chamber using a Pasteur pipet connected to a vacuum line, and wash upper and lower chambers twice with 0.5 and 1.5 ml PBS, respectively.
5. Remove PBS from lower chamber of Transwell. Add 500 μl of 0.25 % Triton X-100 to the upper chamber for 30 min (*see* **Notes 8** and **9**).
6. Transfer cell lysate to a sterile microcentrifuge tube. Using a sterile cell scraper, gently scrape remaining bacteria and cell debris off the upper surface of the Transwell filter. Avoid breaking the filter if possible (*see* **Note 10**). Transfer residual lysate to the sterile microcentrifuge tube.
7. Prepare tenfold serial dilutions of cell lysates 100 μl into 1 ml of LB. Plate 100 μl onto LB plates and incubate overnight to enumerate CFUs.

3.7 Bacterial Adhesion to 3D Cysts

1. After 2 h of infection, remove bacteria in MEM using a Pasteur pipet connected to a vacuum line, and wash three times with 300 μl PBS (room temperature).
2. Fix cells by adding in 300 μl of PBS containing 1 % paraformal-dehyde at 37 °C for 0.5 h.
3. Wash three times with 300 μl PBS (room temperature).
4. When applicable, add primary antibodies (typically 1:300–1:600 dilution) in 300 μl PBS for 2–4 h at room temperature or overnight at 4 °C, and secondary fluorescent antibodies (typically 1:1,000 dilution) in 300 μl PBS at 4 °C overnight.
5. Wash three times with 300 μl PBS (room temperature).

6. Take images with a confocal or fluorescence microscope at a magnification of ×600 or ×1,000.

7. Perform analysis on TIFF files of images taken in the green (488 nm) channel (GFP signal) that contain only bound bacteria scored by eye with Image J: Threshold RGB images using Image > Adjust > Threshold. Score the number and size of bound bacteria (in pixels) using Analyze > Analyze Particles.

3.8 Cytotoxicity Assay

3.8.1 Measurement of Lactate Dehydrogenase (LDH) Release from the Apical Surface of 2-D Monolayers

1. Prior to bacterial infection, replace tissue culture media in the upper chamber of Transwell filter with sterile, pre-warmed MEM.

2. Infect with bacteria for desired length of time, ensuring that the total final volume of media (after bacterial inoculation) in the upper chamber of Transwell filter is 500 μl.

3. At various times after bacterial infection, remove 50 μl of media from the upper chamber of Transwell filter and transfer to a 96-well plate for the cytotoxicity assay.

3.8.2 Measurement of LDH Release from the Basolateral Surface of Cells Grown on Transwell Filters

1. If comparing directly to apical LDH release, replace tissue culture media in the lower chamber of Transwell filter with 500 μl of sterile, pre-warmed MEM. Otherwise, ensure that the lower chamber of all experimental samples contain an equivalent amount of sterile, pre-warmed MEM.

2. At various times after bacterial infection, remove 50 μl of media from the lower chamber of Transwell filter and transfer to a 96-well plate for the cytotoxicity assay.

3.8.3 Positive Control with Lysis Buffer

1. Lyse cells using either the included Lysis Buffer in the CytoTox 96 system or with 0.8 % Triton at room temperature for 45 min. An alternative method of lysis may also be used (*see* **Note 11**).

2. After lysis, remove 50 μl of media from the upper chamber of the Transwell filter and transfer to a 96-well plate for the cytotoxicity assay.

3.8.4 Cytotoxicity Assay

1. For background control, add 50 μl of MEM to three or four empty wells in the 96-well plate.

2. Add 12 ml of RT assay buffer to one vial of substrate mix (*see* **Note 12**). Add 50 μl of reconstituted substrate mix to each culture well. Incubate at room temperature for 30 min and protect from light.
3. Add 50 μl of stop solution to each culture well.
4. Record absorbance at 490 nm (*see* **Note 13**).
5. Subtract background values from the experimental sample readings and positive controls.
6. Determine % cell death using the formula: % cytotoxicity = Experimental LDH release (OD490)/Maximum LDH release (OD490).

4 Notes

1. MDCK clone I and clone II cells have somewhat distinct polarization properties.
2. Calu-3, 16HBE14o-, and CFBE14o-cells tend to clump and require repeated pipetting and vortexing before plating.
3. As there is commonly variability in these assays, it is advisable to use 3–6 replicate wells per sample and condition. In addition, it is useful to use a wild-type bacteria (such as PAK) and an isogenic mutant lacking the two major adhesins, type IV pili and flagella (PAKΔ*pilA*Δ*fliC*) as positive and negative controls. The non-adhesive mutant exhibits at least 100-fold decreased binding compared to the wild-type bacteria.
4. Either log phase or stationary phase bacteria can be used for the adhesion, invasion, or cytotoxicity assays. It is more important that the same bacterial growth conditions are used when repeating assays or when comparing apical to basolateral infections.
5. An OD_{600} of 1 is ~1×10^9 CFU/ml. For infections with MOI of 20, you will need 40×10^6 bacteria (20 times 2×10^6 cells on Transwell filters). For an OD_{600} of 0.4 (~4×10^8 CFU/ml) dilute bacteria 1:10 into 200 μl of MEM.
6. To avoid disrupting the monolayer, place the Pasteur pipet at the edge of the well and slightly above the filter, and tilt the plate to aspirate the media. If any of the epithelial cells in the monolayer are injured, the bacteria will preferentially adhere to this region and the bacteria CFUs in the adhesion assays will be artificially elevated.
7. For *P. aeruginosa* strains that do not harbor antibiotic resistance, either aminoglycoside antibiotic works. If the strain is gentamicin resistant (for example, there is a transposon encoding gentamicin resistance in the genome), then use amikacin.
8. Do not use the lysis method described in Subheading 3.4because this method would include bacteria that adhere to the bottom of the filter.

9. As the cells are lysed by 0.25 % Triton, lysate from the upper chamber may leak through the filter into the lower chamber. This lysate contains bacteria and should be collected as part of **step 6**.

10. It may be helpful to transfer the Transwell inserts to a 6-well plate for the lysis ste. The larger well size allows the user to manually grasp the side of the Transwell insert while using the cell scraper. A firm grip on the Transwell insert will minimize breakage of the filter.

11. Other methods of cell lysis, such as freeze-thaw lysis or mechanical lysis by vortex, may also be used.

12. Reconstituted Substrate Mix may be stored for 6–8 weeks at −20 °C without loss of activity, per manufacturer's instructions (Promega Cytotox 96 Non-Radioactive Cytotoxicity Assay Technical Bulletin, Literature #TB163, revised 12/12).

13. If a large amount of LDH is released in your experimental conditions, positive controls and/or experimental samples may need to be serially diluted with culture medium in order to obtain measurements in the linear range of the plate reader or spectrophotometer.

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