Video Article

In Vitro Assay of Bacterial Adhesion onto Mammalian Epithelial Cells

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Abstract

To cause infections, bacteria must colonize their host. Bacterial pathogens express various molecules or structures able to promote attachment to host cells[1]. These adhesins rely on interactions with host cell surface receptors or soluble proteins acting as a bridge between bacteria and host. Adhesion is a critical first step prior to invasion and/or secretion of toxins, thus it is a key event to be studied in bacterial pathogenesis. Furthermore, adhered bacteria often induce exquisitely fine-tuned cellular responses, the studies of which have given birth to the field of 'cellular microbiology'[2]. Robust assays for bacterial adhesion on host cells and their invasion therefore play key roles in bacterial pathogenesis studies and have long been used in many pioneer laboratories[3,4]. These assays are now practiced by most laboratories working on bacterial pathogenesis.

Here, we describe a standard adherence assay illustrating the contribution of a specific adhesin. We use the *Escherichia coli* strain 2787[5], a human pathogenic strain expressing the autotransporter Adhesin Involved in Diffuse Adherence (AIDA). As a control, we use a mutant strain lacking the *aidA* gene, 2787Δ*aidA* (F. Berthiaume and M. Mourez, unpublished), and a commercial laboratory strain of *E. coli*, C600 (New England Biolabs). The bacteria are left to adhere to the cells from the commonly used HEp-2 human epithelial cell line. This assay has been less extensively described before[6].

Protocol

1. Preliminary: Bacterial strains and epithelial cells.

Manipulations of cells and bacteria are performed aseptically, under a laminar flow hood.

1. Freshly isolate the *E. coli* strains 2787, 2787Δ*aidA*, and C600 from glycerol stocks on Lysogeny Broth (LB) agar plates (1% tryptone, 0.5% sodium chloride, 0.5% yeast extract, 1.5% agar) and grow at 37°C. To minimize variability in the assay, it is advised to always use freshly plated strains and to keep the strains at 4°C on sealed Petri plates only for a maximum of a couple of weeks.
2. Culture HEp-2 cells (ATCC CCL-23) in high glucose Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% heat inactivated bovine serum (heat inactivation is performed at 56°C for 30 min). During routine culture we also add 10 U/ml penicillin and 10 µg/ml streptomycin.
3. Grow Hep-2 cells at 37°C in a cell incubator with an atmosphere containing 5% CO_2. Use standard cell culture procedures to maintain the cells, which are grown in 75 cm^2 flasks and subcultured every time they reach confluence. We use our cultured cells until they reach 30 to 40 passages and then discard them.
4. Prepare an assay when the HEp-2 cells in a 75 cm^2 flask are nearly reaching confluence and therefore are ready for an adherence assay.

2. DAY 1: Preparing the inoculum and the epithelial cells

1. Wash the HEp-2 cells in the flask once with warm Dulbecco's phosphate buffered saline (DPBS: 8 g/L NaCl, 0.2 g/L KCl, 0.2 g/L KH_2PO_4, 0.21 g/L Na_2HPO_4:$7H_2O$)
2. The cells are incubated with 0.05% trypsin - EDTA for 5 min before adding fresh warm complete medium. After fresh medium is added, the cells are resuspended by pipetting up and down.
3. Centrifuge the cell suspension at 2,000 rpm for 5 min and resuspend the pellets in DPBS and centrifuge again.
4. Resuspend the cells in fresh medium supplemented with 10 % serum but containing no antibiotics, at a concentration of $2x10^5$ cells/ml.
5. Seed one milliliter of cell suspension in three sets of duplicate wells (one for each strain) in the center of a 24-well plate and incubate the plate overnight in a cell culture incubator.
   Note: It is important that the serum be thoroughly decomplemented and that antibiotics are omitted after this stage. Failure to do so might kill the infecting bacteria.
6. Inoculate one isolated colony of each bacterial strain (2787, 2787Δ*aidA* and C600) in 5 ml of LB broth (1% tryptone, 0.5% sodium chloride, 0.5% yeast extract) and grow overnight at 37°C with vigorous shaking (180 rpm).

3. DAY 2: Infection of cells

1. Inspect the HEp-2 cells under an inverted microscope to ascertain that they are at least 90 % confluent and not contaminated.
2. Washed the cells with warm DPBS and, to each well, add 1 ml of fresh medium supplemented with 10 % serum but containing no antibiotics. Also add fresh medium to three wells without cells. This will be used to determine the total number of bacteria in the inoculum for each strain.
3. Measure the optical density at 600 nm ($O.D._{600nm}$) of the bacterial cultures. Add an aliquot of each bacterial culture to one set of duplicate wells containing Hep-2 cells and to one well not containing cells. Usually, we use a volume of overnight culture corresponding to 10^6 colony forming units (cfu). This represents a multiplicity of infection (MOI) of 5:1 (bacteria:cells). Although we directly use our overnight cultures, it is

sometimes advisable to centrifuge the bacteria and resuspend them in DPBS to avoid deleterious effects of secreted molecules present in the overnight cultures (such as cytotoxins, for instance)
Note: The MOI can vary between 100:1 and 1:10. Higher MOI yields higher variability and background and bacteria will tend to stick to the plastic of the plate. Lower MOI also yields high variability. Once an MOI is chosen, it is imperative to be consistent and keep this MOI.

4. Incubate the infected cells in the cell culture incubator for 3 hours at 37°C with 5% CO_2.
Note: It is also possible to centrifuge briefly the plate at low speed (e.g. 1,000 x g for 1-2 min), in order to bring all the bacteria directly in contact with the cells. This has the added benefits of synchronizing the infection, and allowing shorter incubation times (as little as 15-30 min).
5. Remove the medium from the infected cells and wash the cells 3 times with warm DPBS. At this step, adherent bacteria can usually be seen with a standard microscope and we routinely perform this check.
6. To lyse the cells and detach the adhered bacteria, add 100 µl of 1% Triton X-100 to each well containing the cells. Other detergents can be used (such as saponin for instance).
7. Incubate the cells for 10 min at room temperature and then add 900 µl of LB medium.
8. Gently homogenize the suspensions by repeated up-and-down pipetting. The wells without cells containing the inoculum are similarly gently homogenized.
Note: Some bacteria might be too sensitive to the detergent, in that case we detach the epithelial cells by incubation with 0.05% trypsin - EDTA for 20 min at 37°C. Cells together with adhered bacteria can also be scraped from the wells with a pipette tip.
9. Prepare serial 10-fold dilutions of the suspensions of adhered bacteria and inoculum using LB broth and plate 100 µl from 3 dilutions (usually the 1:1,000, 1:10,000 and 1:100,000 dilutions) on LB agar and incubate overnight at 37°C.

4. DAY 3: cfu counting and data presentation.

1. Count the colonies on the plates and calculate the number of cfu of adhered bacteria and of the inoculum by averaging each series of dilutions. Only plates with between 10-300 colonies should be counted.

5. Representative results :

The following table shows typical results of cfu counting of adhered bacteria and inoculum from 3 experiments performed on different days:

		Exp. 1		Exp. 2		Exp. 3	
		Dupl. 1	Dupl. 2	Dupl. 1	Dupl. 2	Dupl. 1	Dupl. 2
Strain	Dil	cfu	cfu	cfu	cfu	cfu	cfu
2787 adh.	1,000	389	>300	537	439	562	548
	10,000	66	57	71	62	103	96
	100,000	-	-	-	-	-	-
average cfu		$5.2x10^5$	$5.7x10^5$	$6.2x10^5$	$5.3x10^5$	$8x10^5$	$7.5x10^5$
2787 inoc.	1,000	>300	-	>300	-	>300	-
	10,000	369	-	335	-	451	-
	100,000	38	-	36	-	47	-
average cfu		$3.7x10^6$	-	$3.5x10^6$	-	$4.6x10^6$	-
2787Δadh.	1,000	24	18	19	23	33	29
	10,000	1	1	0	0	2	4
	100,000	-	-	-	-	-	-
average cfu		$1.7x10^4$	$1.4x10^4$	$1.9x10^4$	$2.3x10^4$	$2.6x10^4$	$3.4x10^4$
2787 Δinoc.	1,000	>300	-	>300	-	>300	-
	10,000	402	-	351	-	504	-
	100,000	41	-	38	-	69	-
C600 adh.	1,000	81	68	32	18	47	42
	10,000	3	2	0	0	1	1
	100,000	-	-	-	-	-	-
average cfu		$5.5x10^4$	$4.4x10^4$	$3.2x10^4$	$1.8x10^4$	$2.8x10^4$	$2.6x10^4$
C600 inoc.	1,000	>300	-	>300	-	>300	-
	10,000	309	-	276	-	456	-
	100,000	19	-	27	-	45	-
average cfu		$2.5x10^6$	-	$2.7x10^6$	-	$4.5x10^6$	-

The results can be reported directly as adhered cfu, as shown in Figure 1A. Since the inoculum sizes can vary between strains because of differences in growth rates or pipetting errors, it is often advisable to report the results as percentage of adhered bacteria. The percentage of

adhered bacteria can be calculated by dividing the number of cfu of adhered bacteria by the number of cfu of the inoculum, as shown in Figure 1B. By performing a repeated measure ANOVA and Bonferroni post-tests to compare all columns of Figure 1B, we can see that there are significant differences ($p<0.05$) between 2787 and 2787Δ*aidA*, as well as between 2787 and C600, but no significant difference between 2787Δ *aidA* and C600.

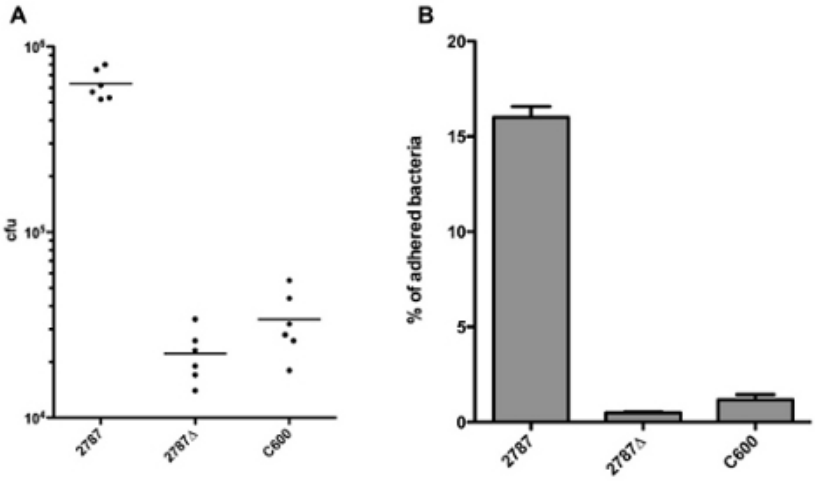

Figure 1. Representation of the results as cfu of adhered bacteria (A) or percentage of adhered bacteria (B)

The percentage of adherence observed is often very dependent on the experimental set-up (and, to a lesser extent, on the experimenter). Particularly important are the MOI and the number of washes, so the percentage should not be literally interpreted.

The bacteria in the inoculum can sometimes grow much faster than the bacteria in the presence of cells, skewing the size of the inoculum by comparison with the real total number of bacteria in wells with cells. To alleviate this issue, it is recommended to either: (i) use wells with cells to determine the inoculum: HEp-2 cells are seeded in an additional well and infected. At the end of the assay, instead of discarding the supernatant and washing, 100 µl of 10% Triton X-100 is added to the well. The cells will lyse and the lysate containing adhered and non-adhered bacteria is gently homogenized by repeated up-and-down pipetting.; or (ii) collect the supernatants of infected cells at the end of the assay, as well as the supernatants from the DPBS washes and determine the number of cfu in those pooled supernatants. This will yield the number of cfu of non-adhered bacteria. The percentage of adhered bacteria can then be calculated by dividing the number of cfu of adhered bacteria by the addition of the number of cfu of adhered and non-adhered bacteria.

To illustrate the robustness of the assay, we can compare this protocol with the assay performed with S4074, an adhesive porcine pathogenic strain of *Actinobacillus pleuropneumoniae*[7], incubated with cells from a recently established tracheal piglet cell line, NPTr[8]. Aside from differences in the media required for growth of the bacteria and the cells, the only difference is that the bacteria used for infection are from an exponentially growing culture, and not from a stationary-phase overnight culture. With *A. pleuropneumoniae* it is also very important to respect an MOI of 10:1 and to not exceed 3 hours of infection, otherwise the secretion of toxins will cause cell death and bias the results. In addition, this strain of *A. pleuropneumoniae* can readily adhere to plastic so it is important to use confluent cells and verify visually that the cells are not sloughing off.

Discussion

This protocol describes a standard bacterial adherence assay that can be modified to study invasion (e.g. using gentamicin protection assay[3]). The counting of colony forming units allows quantification, by comparison with approaches relying on standard visualization techniques under a microscope, such as Giemsa staining. The latter only gives a qualitative view of adhesion but is often a useful complement since it can differentiate various patterns of adhesion and give mechanistical insights[9]. For instance a Giemsa stain performed with an inoculum of 10^7 cfu of 2787 is shown in Figure 2 and indicates a diffuse adhesion on the whole cell surface, characteristic of the Diffusely Adhering *Escherichia coli*[10].

Despite the quantitative aspect of this protocol, it should be emphasized that results are usually variable. There is a fairly constant difference between positive and negative controls, with negative control typically having between 10- and 100-fold lower levels of adhesion than positive controls. But from day to day, the percentage of adhesion can vary up to 2-fold. Therefore it is often advisable to use two to three replicate per experiment, plan several experiments and perform statistical analyses using repeated measures or paired tests (either ANOVA or Student's t test).

Another key point is the washes after adhesion of bacteria. Care must be taken not to detach the epithelial cells. If the cells slough off then there will be less adhered bacteria in that well. More or less washes can be used and the number of adhered bacteria is very correlated with the number of washes (usually between 2 and 4) and depends heavily on how the experimenter performs the washes. The minimum number of washes that allows a low background should be used. No matter how many washes are chosen, it is of utmost importance to employ the same number of washes for all wells and to repeat the same gestures every time a wash is performed.

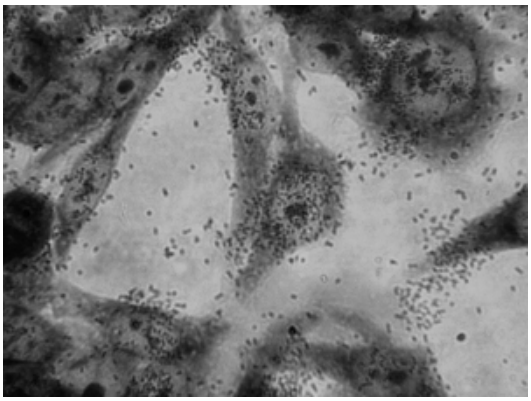

Figure 2. Giemsa staining of bacteria from *E. coli* strain 2787 adhered to HEp-2 cells.

Disclosures

No conflicts of interest declared.

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