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A simple microtiter plate screening assay for bacterial invasion or adherence

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Abstract. Many bacteriologic studies, including cellular invasion and adherence assays, require enumeration of viable organisms or colony forming units. When attempting to screen large numbers of clinical or environmental isolates or laboratory-derived mutants for differences in invasion or adherence phenotype, standard plating methods can be cumbersome and severely limit the number of organisms or conditions which can be tested. As a potential alternative, we describe a simple, rapid and inexpensive soft agar-based technique for semi-quantitative determination of bacterial colony counts directly within the wells of a 96-well microtiter plate.

Key words: Bacterial adherence, Bacterial invasion, Bacteriological techniques, Invasion assay, Microbial colony count, Soft agar

1. Introduction

Many bacteriologic studies, including cellular invasion and adherence assays, depend at some point on data derived from enumeration of viable organisms or colony forming units. When screening large numbers of clinical or environmental isolates or laboratory-derived mutants in such quantitative assays, standard plating methods can be time-consuming and limit the number of organisms or conditions which can be tested. Alternative methods to agar plating for bacterial quantification include: direct microscopic enumeration in counting chambers [6], epifluorescence microscopy using acridine orange or other fluorochromes [3, 4], flow cytometry [8], impedance bacteriometry [5], tetrazolium dye reduction [7], measurement of adenosine triphosphate bioluminescence [10], and enzyme-labeled, rRNA-targeted oligonucleotide probes [1]. None of these methods, however, allows the investigator to assay bacterial viability, antibiotic susceptibility and metabolic phenotype simultaneously, as can be done with growth on specialized agar media.

In the course of our studies of cellular invasion and adherence by group B streptococci (GBS) and other bacterial pathogens, we have employed a simple, rapid and cost-saving soft agar technique for the semi-quantitative determination of bacterial colony counts directly within the wells of 96-well microtiter plates. Although we hesitate to claim that a similar technique has never been described, the current literature in the field of bacterial pathogenesis indicates that standard labor-intensive plating techniques are widely used, even when great numbers of isolates or laboratory-derived mutants are being screened. Therefore, we offer a brief description of our screening invasion and adherence assays, in hope that others may find a useful application for the soft agar technique in their own experiments.

The screening cellular invasion assay is adapted from the antibiotic protection procedure first described by Devenish and Schiemann [2]. Certain antibiotics (e.g., gentamicin) will effectively kill extracellular or surface-adherent bacteria, but inefficiently penetrate eukaryotic cells so as not to reach microbicidal levels in the intracellular compartment. The differential antibiotic killing can be exploited to quantify the degree of invasion of viable bacteria into the host cell. Previously, we had quantified the number of internalized bacteria by plating small aliquots of an epithelial cell lysate onto standard agar petri dishes containing the appropriate growth media. When screening large numbers of transposon mutants for modifications of invasion phenotype, however, we found plating samples from each individual well to be quite tedious. A single person could screen comfortably no more than 100 mutants in a single work day. Moreover, the inherent time delay between plating of the first and last samples introduced an undesired variable into determination of viable counts, and replicate samples often demonstrated differences attributable to sampling or plating error. For screening purposes, semi-quantitative discrimination of colony forming units would suffice. We therefore

modified our protocol to eliminate the labor-intensive plating steps, and employed soft agar to enumerate the bacteria directly within wells of a 96-well microtiter plate. We subsequently adapted this methodology to develop a screening assay for streptococcal adherence to human platelets.

2. Materials

1. 96 well tissue culture plates, flat bottom, sterile with lid (Costar #3598).
2. Todd Hewitt Broth (Difco #DF0492-17-6).
3. Bacteriologic grade agar (Difco DF0140-01-0).
4. A549 human lung carcinoma cell line (ATCC #CCL-185).
5. RPMI 1640 tissue culture media, with HEPES (Sigma #R 6504).
6. Fetal calf serum, heat inactivated (Sigma #F 4135).
7. 12-channel pipettors (Labsystems #4510040 and 4510050).
8. Boekel 96-pin stainless steel replicator (Fisher #05-450-9).
9. Reagent reservoir (Sigma #R 1936).
10. Bent stainless steel manifold (Drummond #3-00-094).
11. Gentamicin sulfate (Sigma #G 1264).
12. Penicillin G (Sigma #P 7794).
13. 0.25% Trypsin/EDTA solution (Sigma #T 4049).
14. Triton X-100 (Sigma #X-100).
15. Poly-L-Lysine (Sigma #P 4832).
16. Brain-Heart Infusion (BHI) Broth (Difco DF0037-07-0).
17. Tyrode's salt solution (Sigma #T 2397).

3. Procedures

A. *Cellular Invasion Assay*

1. *Preparation of bacteria and tissue culture cells*
   A549 lung epithelial cells are seeded and grown in tissue culture media (RPMI media + 10% fetal calf serum) in 96-well tissue culture plates until confluent monolayers are formed. The day before the screening assay, single colonies of GBS mutants are picked with a sterile toothpick and used to inoculate 200 µl of Todd Hewitt broth (THB) in individual wells of 96-well microtiter plates. Control wells on each plate are inoculated with either the wild-type GBS strain or a noninvasive bacterial isolate (e.g., *Escherichia coli* strain DH5 or *Streptococcus gordonii* Challis). The plates are incubated overnight at 37 °C to allow the bacteria in each well to reach stationary growth phase. Cellular invasion by certain bacterial species may require factors expressed only during exponential growth phase. In such cases, a replica plate with 200 µl of fresh media per well may be inoculated with 10 µl of the overnight cultures and growth monitored by optical density measurement using a microplate reader.
2. *Inoculation of cell monolayers*
   Immediately prior to the invasion assay, each plate is vortexed gently to resuspend settled bacteria. Optical density measurement can be obtained to identify any strains or mutants which grew poorly, such that any spuriously low input inoculum can be considered in interpretation of final invasion assay results. Using an 8- or 12-channel pipettor, 10 µl of each bacterial culture is transferred as an inoculum to a corresponding well in a 96-well plate containing 200 µl antibiotic-free tissue culture media and a confluent A549 cell monolayer. Alternatively, the plates may be inoculated using a Boekel 99-pin stainless steel replicator which transfers a standard volume of the liquid culture by capillary action.
3. *Antibiotic protection method for determination of cellular invasion*
   Following inoculation, the 96-well tissue culture plates are centrifuged at 800 ×*g* for 10 min to place GBS at the surface of the cell monolayer, then incubated for 2 h at 37 °C with 5% CO_2 to allow cellular invasion by the GBS. After the incubation, medium is removed from the monolayers by gentle aspiration of the wells using a bent-hub 8-channel stainless steel manifold attached to a vacuum source. The subsequent wash and treatment steps all involve liquids dispensed from a sterile reagent reservoir. Monolayers are washed ×3 by adding 200 µl of phosphate buffered saline (PBS) via a multichannel pipettor, followed by gentle aspiration of the wash buffer with the vacuum manifold. Alternatively, removal of medium or wash buffer can be accomplished by rapid inversion and brief shaking of the plate over a sink or disposal container. After the primary wash steps, 200 µl of tissue culture medium containing 100 µg/ml gentamicin and 5 mg/ml penicillin G is added to each well, and the plates incubated for 2 h at 37 °C with 5% CO_2 to kill extracellular and surface-adherent bacteria. The monolayers are once again washed ×3 with PBS. Finally, 50 µl of a 1:4 mixture of 0.25% trypsin/EDTA solution and 0.025% Triton X-100 is added to each well, the plates incubated for 10 min at 37 °C in order to disrupt the epithelial cell monolayers and liberate intracellular bacteria.
4. *Semiquantitative determination of bacterial colony counts using soft agar*
   Todd Hewitt soft agar (THSA) medium is prepared in advance by using 0.7% Bacto

agar (Difco) rather than our standard 1.5%, maintained in liquid form by incubation in a 45–50 °C water bath, and dispensed into a reagent reservoir just prior to use. Addition of 150 μl of THSA to each well using the multichannel pipettor results in uniform dispersion of the 50 μl lysate and solidification within a few minutes at room temperature. The microtiter plates are incubated overnight at 37 °C, and the next day bacterial colonies are observed growing within the THSA. Identification of isolates or mutants that are invasive (some colonies present), hyperinvasive (many colonies present) or noninvasive (no colonies present) is easy and reproducible. Mutants with suspected alterations in invasion phenotype are subsequently confirmed by means of a standard 24-well plate quantitative cellular invasion assay [9].

B. *Platelet adherence assay*

1. *Preparation of bacteria and platelet monolayers*

   To identify transposon mutants of *Streptococcus sanguis* deficient in binding to human platelets, we have performed a similar 96-well microtiter plate screening assay. Briefly, washed human platelets are obtained from fresh blood donated by healthy volunteers, isolated by centrifugation (100 ×*g*, 15 min), followed by washing, fixation in 0.8% formaldehyde (30 min at 37 °C), additional washing, and suspension in Tyrode's solution. Platelet monolayers are prepared by placing 10^7 fixed platelets into each well of 96-well tissue culture plates pretreated with poly-L-lysine (0.01% solution).

2. *Semiquantitative determination of platelet binding using soft agar*

   Overnight cultures of *S. sanguis* mutants are grown in BHI broth, washed and resuspended in Tyrode's salt solution. One hundred μl of each bacterial suspension is transferred (~10^7 cfu) onto the immobilized platelets in a corresponding microtiter well and rocked gently at 4 °C for 1 h. The unbound organisms are then removed by washing three times with Tyrode's solution. The wells are treated with trypsin (1 mg/ml, 10 min) to release the adherent bacteria. Brain-Heart Infusion soft agar (BHISA) medium is prepared in advance by using 0.7% Bacto agar (Difco), maintained in liquid form by incubation in a 45–50 °C water bath, and dispensed into a reagent reservoir just prior to use. Using a multichannel pipettor, 175 μl of BHISA is added to each well. After overnight incubation at 37 °C, the number of organisms per well is assessed qualitatively by visual inspection. Those wells with markedly decreased quantities of colony forming units represent possible low-platelet-binding mutants, whose phenotype is subsequently confirmed by a quantitative platelet binding assay [11].

4. Results and discussion

Figure 1 demonstrates GBS recovered within microtiter wells by the soft agar technique following invasion of lung epithelial cell monolayers. Serial twofold dilutions of the organism were used as initial inoculums over a range at which invasion is approximately linear, in order to demonstrate the observed colonial morphology and reproducibility of the assay. Each dilution was performed in triplicate, and three replica wells were assayed by spread plating on Todd-Hewitt agar in standard petri dishes. After overnight incubation, high colony counts imparted a fine granular appearance to the solidified soft agar, whereas at low colony counts, larger discrete colonies were evident. Below approximately 1,000 colonies per well, even two-fold differences in colony number were easily discernable. For quantitative comparison, the colony forming units counted on the spread plates for each dilution were as follows (+ st dev): 1260 + 80, 635 + 32, 301 + 34, 164 + 20, 79 + 4, 42 + 6, 19 + 6, 12 + 8, and 4 + 2.

Figure 2 illustrates how a low platelet binding mutant of *S. sanguis* can be readily identified by visual inspection following the semiquantitative soft agar-based screening platelet adherence assay. A similar screening assay allowed identification of low platelet binding variants of *Staphylococcus aureus* [11]. In additional experiments using appropriate soft agar media, we have successfully adapted our method to assay the invasion potential of *Escherichia coli*, *Burkholderia cepacia* and *Haemophilus influenzae*. The soft agar microtiter plate technique should be generalizable to a host of cellular adherence and invasion assays, microbicidal assays and numerous other bacteriologic experiments which can be performed in a microtiter well and require only semiquantitative interpretation. Once a particular assay is developed, 'standard curves' from known serial dilutions of the organism grown in soft agar media can be constructed and used to assign approximate numerical values to matching experimental wells.

We have found several advantages to the use of the soft agar microtiter well assay as compared to standard bacteriologic plating methods. First, the entire well is assayed for colony forming units, eliminating both sampling error in obtaining a small aliquot and plating error associated with 'hockey-sticking' or other dispersion techniques. Second, the assay is extremely rapid, requiring but seconds for delivery of the soft agar with a multichannel pipettor. Severalfold more samples can be screened efficiently in a given experiment, and intersample variation,

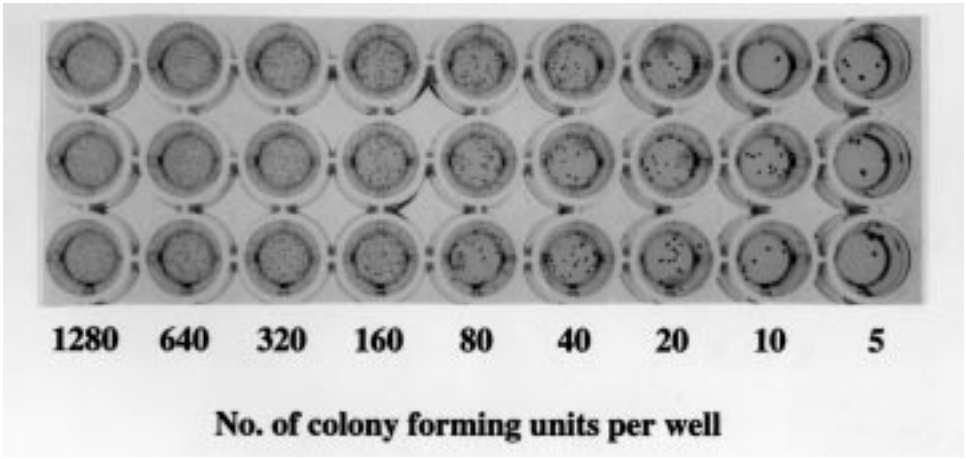

Figure 1. Semi-quantitative invasion assay in which serial dilutions of group B streptococci are added to lung epithelial cell monolayers in microtiter wells, extracellular bacteria eliminated by washing and gentamicin treatment, and intracellular bacteria recovered by addition of soft agar and overnight incubation at 37 °C. The experiment was performed in triplicate and colony counts confirmed by standard plating methods.

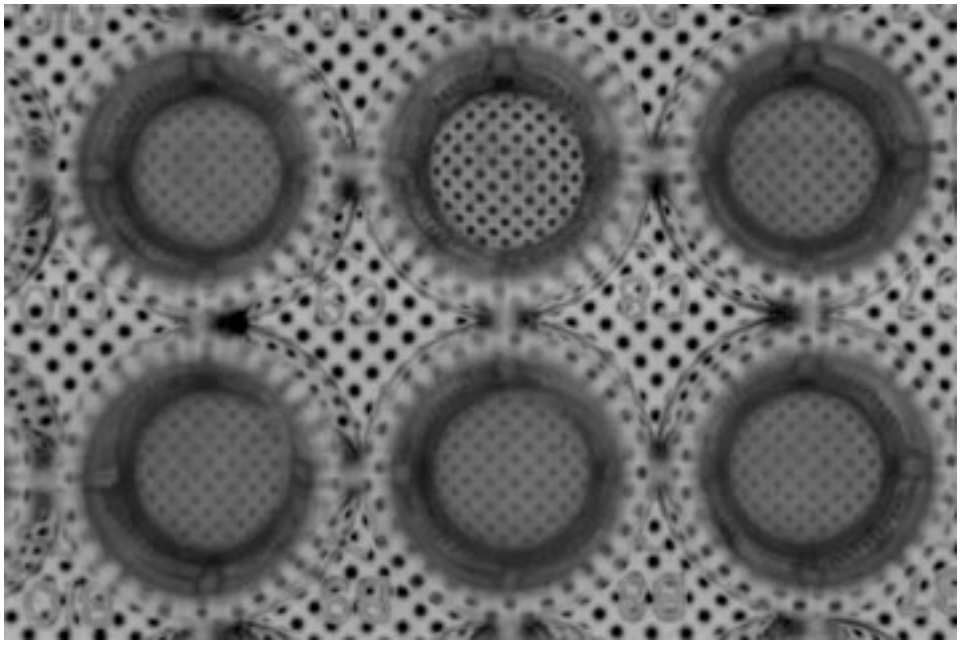

Figure 2. Use of soft agar to identify low platelet binding variants of Streptococcus sanguis M99. One of the center wells is much clearer, indicating greatly decreased colony forming units associated with low binding to platelets immobilized on the microtiter well surface.

due to time elapsed between individual platings, is avoided. Third, the assay is easy to interpret, since positive and negative controls can be placed on the same plate for direct visual comparison with sample wells. Lastly, the assay requires almost no additional expense, in contrast to the considerable cost and/or labor involved in preparing large numbers of standard agar petri dishes. Stocks of soft agar medium can be made in advance, allowed to solidify, and the required amount melted using a microwave on the day of the assay.

All soft agar assays should be calibrated such that <1,000 colony forming units are typically recovered in a single well; alternatively, appropriate dilution into a replica microtiter plate can be performed prior to addition of the soft agar. We recognize that a soft agar microtiter well assay may not be applicable to organisms requiring high oxygen tensions and those acutely sensitive to the initial temperature elevation required to maintain the agar in liquid form. Formation of large or confluent surface colonies would also tend to obscure organisms embedded within the agar. Nevertheless, we encourage other investigators interested in large scale screening of microorganisms for viable colony counts to consider this semi-quantitative technique. By delivering the

agar to the organism (rather than the other way around), substantial laboratory time, effort and expense may be saved.

Acknowledgments

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Notes on suppliers

1. Corning Costar Corporation, One Alewife Center, Cambridge, MA 02140, USA
2. Difco Laborotories LTD, P.O. Box 14B, Central Avenue, West Molesey Surrey, England, KT8 2SE
3. Drummond Scientific Co., 500 Parkway Blvd., Broomal, PA 19008, USA
4. Fisher Scientific, 711 Forbes Avenue, Pittsburgh, PA 15219, USA
5. Labsystems Oy, P.O. Box 8, FIN-00881 Helsinki, Finland
6. American Type Culture Collection (ATTC), 12301 Parklawn Drive, Rockville, MD 20852, USA
7. Sigma, 3050 Spruce Street, St. Louis, MO 63103, USA

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