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Quantitation of HeLa Cell Monolayer Invasion by *Shigella* and *Salmonella* Species

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A major determinant in the virulence of *Salmonella* and *Shigella* spp. is the ability of these organisms to invade epithelial cells of the gastrointestinal mucosa and multiply intracellularly. The invasion of cell culture monolayers is a convenient experimental system to evaluate eucaryotic cell penetration and is correlated with the potential of a strain to cause human disease. We have developed an agarose-L agar overlay technique which allows for the convenient quantitation of the number of infected tissue culture cells in a monolayer. Bacterial strains were introduced onto antibiotic-free HeLa cell monolayers. Infected monolayers were washed, and noninternalized bacteria were counterselected with kanamycin (50 μg/ml). The number of infected HeLa cells present was determined by overlaying the monolayer with distilled water-agarose (0.5 to 1.5%) followed by an equal volume of 2× L agar. Bacterial colonies formed over infected cells in 24 h at 37°C, and wells were counted with a dissecting microscope under ×2 power. Bacterial colonies were not observed with noninvasive variants of *Shigella* spp. To obtain countable wells (20 to 200 CFU) the multiplicity of infection or invasion times were adjusted. With a 90-min invasion time, the invasive potential of a strain was reflected by the multiplicity of infection needed to produce countable wells. The quantitation of bacterium-invaded cells by using standard bacteriological methods is a convenient and rapid method to evaluate the invasive potential of bacterial strains. Additionally, parameters essential for the invasive process can easily be investigated.

The ability of enteroinvasive bacteria to invade cell culture monolayers has been used as a convenient measure of the invasive potential of a bacterial strain. The ability of *Shigella* spp. (9, 14, 16), *Salmonella* spp. (3, 4, 7, 10), invasive *Escherichia coli* (2, 15), and to a lesser extent *Yersinia enterocolitica* (23) to invade cell culture monolayers is highly correlated with their potential to produce disease in humans. Indeed, *Shigella flexneri* and *S. sonnei* strains which lose the ability to penetrate HeLa cells are uniformly avirulent in animal hosts (8, 19–21). The invasive phenotype has been mapped to a 140-megadalton extrachromosomal element (6, 8, 19–21) with spontaneous deletions in the plasmid, leading to noninvasive variants (12). In *Salmonella typhimurium* strains, Giannella et al. (3, 4) have shown a correlation between penetration of HeLa cells and virulent activity in rabbit intestinal loops. In short, the use of cell culture monolayer penetration represents a convenient experimental system in which conditions are easily controlled or varied.

The most difficult problem in assessing cell culture monolayer penetration is the determination of which bacteria are intracellular and which are residing between cells, attached to cells, or attached to the tissue culture plasticware. To quantitate intracellular bacteria, studies have involved the use of direct microscopic examination by specific or nonspecific staining techniques (3–6, 8, 9, 12, 14, 16, 19–21) or procedures in which intracellular bacteria are enumerated by plating (1). Both procedures involve the use of methods to remove or reduce adherent noninternalized bacteria. Hale and Bonventre (5) and others (7, 16) have used antibiotic counterselection to remove adherent bacterial populations. For these methods, antibiotics which sparingly cross eucaryotic cell membranes are used as the counterselective agent. As an applicable alternative, Shaw et al. (22) have used UV-irradiated bacteriophage T6 to lyse noninternalized bacteria.

Microscopic examination of infected cells relies on the ability to discriminate between noninternalized bacteria and intracellular bacteria which lie in the same focal plane as cellular ultrastructures. Kihlström (7) has used specific staining with immunofluorescent bacterium-specific antibodies to distinguish intracellular from adherent bacteria after eucaryotic membrane solubilization. Although microscopic examination yields the number of cells infected and the number of internalized bacteria, the method is cumbersome and slow. Furthermore, this method does not identify viable internalized bacteria, and low levels of infection may not be discernible.

An alternative method to quantitate internalized bacteria involves disruption of the infected monolayers and plating of the resulting lysate. Bhogale et al. (1) have used this method to quantitate invasion by *Salmonella* and *Shigella* spp. This method enumerates the invasive bacteria and distinguishes those bacteria capable of outgrowth, but cannot enumerate the infected cells in the monolayer.

In this study, we describe an agarose-L agar overlay procedure which allows rapid and convenient quantitation of the invasion of cell culture monolayers. Outgrowth of bacteria from overlaid tissue culture cells allows us to identify the number of infected cells as well as the invasion efficiency of the bacterial strain.

MATERIALS AND METHODS

Bacterial strains. Invasive and noninvasive strains of *Salmonella* and *Shigella* spp. were used. *S. typhimurium* TML, W118, and SL1027 have been used in previous studies (3, 4). *S. flexneri* SA100 has been recently described (18). The invasiveness of *S. flexneri* M90Tvir$^+$ has been well characterized, and M90Tvir$^-$ is an isogenic noninvasive variant of M90Tvir$^+$ which has been cured of the 140-megadalton plasmid (6, 19, 21). *S. sonnei* 1694 and *S.*

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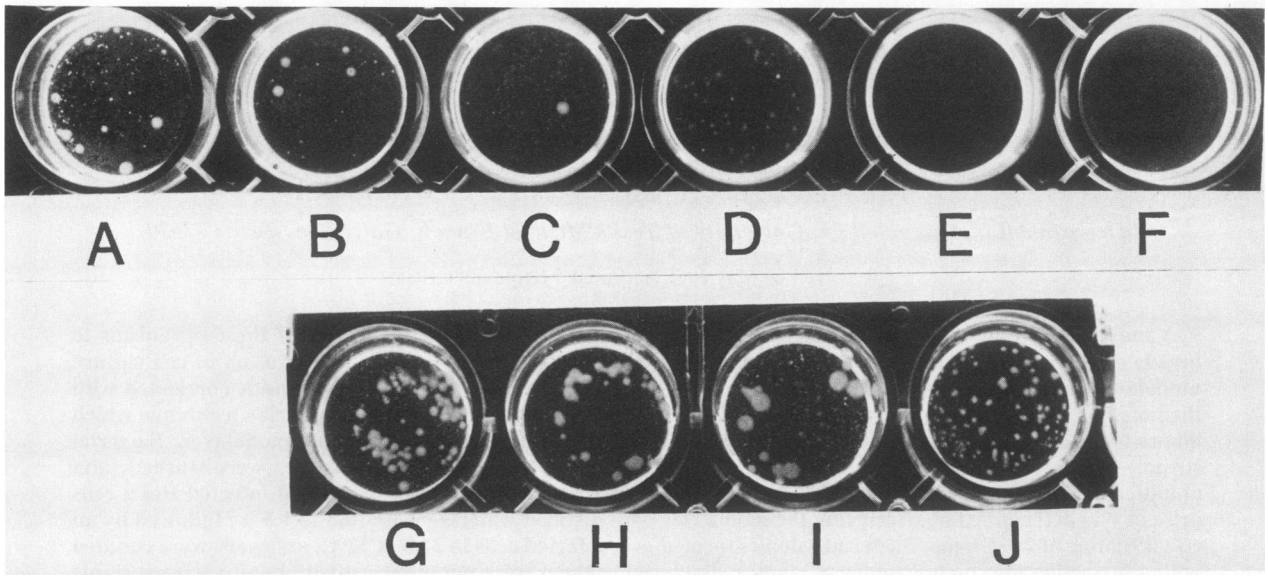

FIG. 1. Top. Treatment of HeLa cell monolayers with *S. flexneri*. Strains SA100 and SA100NI were prepared as described in Materials and Methods. Well A was treated with SA100, 2.5×10^8 cells (MOI, 3,000/l); well B contained 2.5×10^7 cells (MOI, 300/l); well C contained 2.5×10^6 cells (MOI, 30/l); well D contained 2.5×10^5 cells (MOI, 3/l); well E contained SA100NI, 1.1×10^8 cells (MOI, 1,300/l); well F was mock infected. Bacterium/HeLa cell ratios were determined by assuming the presence of 8×10^4 HeLa cells per well. Monolayers were overlaid after 4 h of counterselection and incubated overnight. Bottom. Invasion of HeLa cell monolayers by *S. typhimurium* SL1027. The procedure was as indicated above, except monolayers were overlaid with 1.5% agarose in distilled water and 2× L broth with 1.5% agar. Well G was treated with SL1027, 1.1×10^6 cells (MOI, 125/l); well H contained 1.1×10^5 cells (MOI, 12.5/l); well I contained 1.1×10^4 cells (MOI, 1.25/l); well J contained 1.1×10^3 cells (MOI, 0.125/l). Punctate colonies are those on the bottom of the wells, whereas large, globular colonies are surface colonies. Colonies appearing in the overlays are easily distinguished by their disk-shaped appearance.

dysenteriae 1-130 were obtained from S. Payne, University of Texas, Austin. All other strains were obtained from G. Buck, Clinical Services Department of John Sealy Hospital, University of Texas Medical Branch, Galveston. Noninvasive isogenic variants were isolated from *S. flexneri* SA100 by picking white colonies on Congo red agar by the procedures of Maurelli et al. (12) and Payne and Finkelstein (17). Noninvasive variants of *S. flexneri* were selected as fresh isolates monthly. Strains were maintained as stocks in 20% glycerol–tryptic soy broth (Difco Laboratories) at −70°C. Strains were passed on L agar and started from frozen stocks monthly. For invasion studies, strains were grown overnight in L broth and split 1/100 into brain heart infusion broth (Difco). Bacterial strains were grown to mid-logarithmic phase (optical density at 600 nm, 0.6), washed, and suspended in RPMI 1640–0.67 μg of $FeCl_3$ per ml–0.45% glucose (RPMI Fe), as described by Oakes et al. (13), in preparation for invasion.

Cell culture methods. Briefly, HeLa cells were maintained as monolayers in supplemented RPMI 1640 (5% fetal bovine serum, 2 mM glutamine, 50 U of penicillin G, 50 μg of streptomycin per ml) in a 5% CO_2 humidified atmosphere. Cells were trypsinized to detach them from the plastic flasks and seeded into 24-well plates at 8×10^4 per well. This concentration gave a nearly confluent monolayer of HeLa cells in 24 h. Antibiotic-free medium of the same composition was used to wash the monolayers before the invasion studies were begun.

Invasion of HeLa cell monolayers. Bacterial strains were diluted into RPMI Fe with no antibiotics. Cells were maintained at 37°C or room temperature. Medium was removed from HeLa cell monolayers and replaced with 250 μl of bacteria in RPMI Fe. After invasion, bacteria were removed by aspiration and washed six times with wash medium consisting of 67% Hanks balanced saline solution and 33% RPMI 1640 supplemented with 5% fetal bovine serum and 50 μg of kanamycin per ml. Plates were shaken gently on a rotating platform (100 rpm; Queue model 4730) for 1 min between washes. This washing procedure produced monolayers which microscopically appear free of adherent bacteria. Noninternalized bacteria were selected against by replacing the final wash solution with RPMI Fe–50 μg of kanamycin per ml–5% fetal bovine serum for various times as indicated in the figure legends. After counterselection, medium was removed and the monolayers were gently rinsed with RPMI Fe to remove residual antibiotics. This medium was removed, and HeLa cells were lysed by the addition of 250 μl of sterile 0.5% agarose–distilled H_2O maintained at 45°C. A 250-μl portion of 0.5% 2× agar was layered over the solidified agarose and allowed to harden at room temperature. For *S. typhimurium* strains, agarose and agar were used at a higher concentration (1.5%). Plates were returned to 37°C and incubated overnight. Plates were counted with the aid of a dissecting stereo microscope.

RESULTS

Preliminary experiments with each strain were performed to determine the multiplicity of infection (MOI) which gave wells with approximately 20 to 200 colonies. This corresponds to an infection rate of 0.02 to 0.2% based on 8×10^4 HeLa cells per well. This number of colonies was easily counted in 24-well plates with the aid of a stereo dissecting microscope. Once established, a MOI consistently gave countable wells when a given strain was used. For *Shigella* strains, overlays with 0.5% agarose–2× L agar gave well-defined colonies above HeLa cell ghosts. For *S. typhimurium* strains which were motile, agarose-agar concentrations were increased to 1.5% to prevent spreading. An

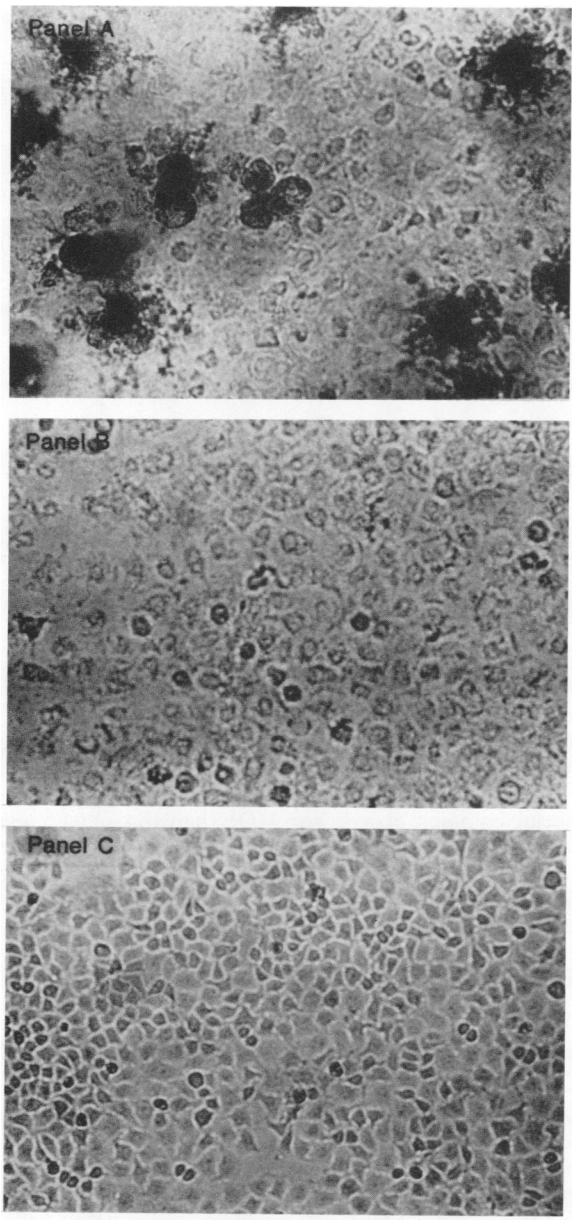

FIG. 2. Appearance of lysed monolayers after treatment with *S. flexneri* SA100 and SA100NI and agarose-agar overlaying. Strains SA100 and SA100NI were prepared as described in Materials and Methods. Wells were treated for 40 min with SA100 (8×10^6 cells; MOI, 100/l), SA100NI (8×10^6 cells; MOI, 100/l), or were mock infected. Wells were washed, noninternalized bacteria were removed by 4 h of counterselection with 50 μg of kanamycin per ml, and monolayers were overlaid as described in the text. Monolayers were observed under ×100 power with an inverted microscope at 18 h after overlaying. Panel A (top) shows monolayer treated with invasive SA100 cells. Opaque areas are bacterial colonies which have emerged from the ghosts of infected HeLa cells. In all instances, colonies arise from a single or closely adjacent HeLa cell. Panel B (middle) shows monolayers treated with SA100NI cells. No opaque bacterial colonies are observed, indicating the efficiency of the washing and antibiotic counterselection of remaining noninternalized bacteria. Panel C (bottom) shows the overlaid mock-infected monolayer for comparison.

agarose-distilled H_2O overlay slightly enhanced colony recovery. However, the major effect of the agarose overlay was in the formation of well-defined punctate colonies directly over infected HeLa cells.

Treatment of HeLa cell monolayers with invasive *Shigella* spp. for 90 min gave rise to distinct colonies in L agar-agarose overlays (Fig. 1). The number of colonies recovered was shown to be dependent on the MOI ratio used (wells A through D) (i.e., 43 punctate colonies in well D). For *S. flexneri* SA100, a MOI in the range of 5/1 to 100/1 with a 90-min invasion time consistently gave countable wells. Isogenic noninvasive variants of strain SA100 (SA100NI) at a higher MOI failed to form colonies under these conditions (well E). The inability of noninvasive strains to produce colonies after overlaying is probably the result of antibiotic-mediated killing. Mock-infected cells served as contamination controls and were included in all plates (well F).

The essential question was whether the number of colonies recovered represented the number of infected cells in the monolayer. The outgrowth of *Shigella* spp. from infected cell culture monolayers is shown in Fig. 2. In cell culture monolayers treated with virulent *Shigella* spp., bacterial outgrowth was observed to occur only above or within ghosts of lysed HeLa cells (panel A). Bacterial colonies arose exclusively from within HeLa cell ghosts for a wide range of invasion times (5 to 300 min) and counterselection times (0 to 4 h). The occasional distribution of colonies through or on the surface of the overlay did not, in most instances, preclude the enumeration of infected HeLa cells. Colonies arising from HeLa cell ghosts were easily distinguished as punctate colonies on the bottom of the well. Surface colonies were easily distinguished by their characteristic shape on the surface of the agar. For example, four surface colonies are observed in Fig. 1, well B, and a single surface colony is observed in well C. Other surface colonies are observed in well A and in wells G, H, and I. Colonies growing through the overlay were disk shaped and are easily distinguished with a dissecting microscope. We have observed that longer invasion times or longer counterselection periods, or both, led to significantly higher bacterial dispersal in the overlays. This could result from intracellular bacterial replication which leads to cellular fragility and lysis of the infected cell during the overlaying procedure. In this regard, we have counted the total number of invasive bacteria by the lysing of infected monolayers and plating procedures of Bhogale et al. (1). These results indicated an average of 10 to 12 *Shigella* cells per infected HeLa cell after 4 h of counterselection and 30 to 35 *Shigella* cells per infected HeLa cell after overnight counterselection.

As noted previously, monolayers infected with noninvasive variants or mock-infected monolayers failed to form colonies in the overlays. In panels B and C, opaque clusters in HeLa cell ghosts, associated with bacterial colony formation, were not observed (Fig. 2). This further indicated the inability of noninternalized bacteria to form colonies in the overlays. The invasion of tissue culture monolayers by *S. typhimurium* SL1027 is shown in Fig. 1, wells G through J. In contrast to that of *S. flexneri* SA100, a MOI ratio of 0.125 gave wells which could be enumerated (well J). *S. typhimurium* strains failed to form punctate colonies at <1% agarose concentrations.

The effect of different concentrations of kanamycin on the recovery of bacterial colonies from infected HeLa cells is shown in Table 1. As shown, kanamycin concentrations greater than 5 μg/ml prevented colony formation in washed wells treated with as many as 2×10^7 noninvasive *S. flexneri*

TABLE 1. Effect of kanamycin concentration on bacterial colony formation

S. flexneri strain	Kanamycin concn (μg/ml)	No. of colonies[a]
SA100	0	47 ± 4
	5	45 ± 5
	10	36 ± 3
	25	49 ± 5
	50	40 ± 11
SA100NI	0	16 ± 22[b]
	5	4 ± 4[b]
	10	0 ± 1
	25	0 ± 1
	50	0 ± 0

[a] Values represent the average of three wells.
[b] Colonies arising from strain SA100NI should represent bacterial outgrowth from noninternalized cells not removed by antibiotic counterselection.

SA100NI cells (MOI, 250/1). No reduction in bacterial colonies arising from internalized invasive strain SA100 was observed after 4 h of counterselection with 5 to 50 μg of kanamycin per ml, suggesting that kanamycin concentrations in this range do not affect the recovery of internalized bacteria in overlays. Since 50 μg of kanamycin per ml prevented colony formation by all noninvasive *Shigella* spp. tested but did not affect the recovery of internalized bacteria, 50 μg of kanamycin per ml was used as the counterselective agent in all subsequent experiments.

Table 2 shows the concentration (MOI) and time dependence of colony formation for *S. flexneri* SA100. For any given MOI, the formation of bacterial colonies was dependent on the invasion time. For example, six colonies were observed after 90 min of invasion, at a MOI of 5.6/1, 41 were observed after 180 min, and after 300 min the colonies were too numerous to count. Additionally, the number of colonies increased in a dose-dependent manner with the number of infecting bacteria for any invasion time. Therefore, one can obtain wells with countable colonies for enumeration by adjusting MOI ratios and invasion times. Occasionally, colonies arising from wells treated with noninvasive strains were obtained at very long invasion times. These colonies were noninvasive variants on the basis of their phenotypes. Their protection from the antibiotic counterselection is unknown.

In Table 3, bacterial colony overlays were used to quantitate bacterial invasion by a number of bacterial species. For *S. flexneri*, a MOI of 0.3 to 75 resulted in wells with 20 to 200 colonies (0.02 to 0.2% infection). As shown earlier, noninvasive variants (M90Tvir$^-$) did not lead to colony formation at a MOI 10 times higher than that of the invasive strains. For *S. sonnei*, the MOI was greater, ranging from 7.5 to 630, with noninvasive form II colonies failing to give bacterial outgrowth. For *Shigella* spp., recent clinical isolates (i.e., 7342, 5348, 4283, and 4162) invaded and produced bacterial colonies at a lower MOI than did older isolates (M90Tvir$^+$ and 1694). Invasion by the *S. dysenteriae* strain tested was comparable to that observed for *S. sonnei* and *S. flexneri* strains. Invasion by *S. typhimurium* generally occurred at lower MOIs than for *Shigella* spp. (0.014 to 0.38). These overlays required the use of 1.5% agarose–agar to prevent spreading and maintain distinct colonies.

TABLE 3. Invasion[a] of HeLa cell monolayers in standard assay

Organism	Strain	No. of bacteria/ HeLa cell	CFU/well[b]
S. flexneri	7342	0.3	211 ± 6
	5348	9	205 ± 6
	7908	75	20 ± 5
	M90Tvir$^+$	50	81 ± 3
	M90Tvir$^-$	500	0 ± 0
	SA100vir$^+$	125	74 ± 10
	SA100NIvir$^-$	900	0 ± 0
S. sonnei	7963	9.4	89 ± 3
	4283	7.5	94 ± 9
	7961	62.5	137 ± 8
	1694	630	11 ± 1
	4162 form I	60	225 ± 16
	4162 form II	1,250	5 ± 2
S. dysenteriae	1–130	75	42 ± 11
S. typhimurium	TML	0.4	69 ± 11
	SL1027	0.4	33 ± 6
	W118	0.09	32 ± 1

[a] Bacterial strains, HeLa cells, and invasions were as described in the text. Counterselection was for 4 h.
[b] Values represent the average of at least duplicate wells.

DISCUSSION

The ability of enteric pathogens to invade cells of the intestinal mucosa is an essential step in the pathogenesis of bacillary dysentery disease. Giannella et al. (3, 4) have shown that *S. typhimurium* strains which are deficient in the ability to invade epithelial cells fail to cause disease in animals or provoke characteristic pathogenesis in ligated rabbit ileal loops. Similarly, invasion of epithelial cells by *Shigella* spp. has been shown to be a critical event in orally

TABLE 2. MOI and time-dependent colony formation in HeLa cell monolayers

S. flexneri strain	No. of bacteria	MOI	CFU/well after invasion[a]:		
			90 min	180 min	300 min
SA100 (*inv*$^+$)	5×10^4	0.56/1	0	7.5	57
	5×10^5	5.6/1	6	41	TNTC
	5×10^6	60/1	74	500	ND[b]
	5×10^7	600/1	TNTC[b]	TNTC	ND
	5×10^8	6,000/1	TNTC	TNTC	ND
SA100NI (*inv*$^-$)	7.5×10^7	1,000/1	0	0	2

[a] Noninternalized bacteria were removed by 4 h of counterselection with 50 μg of kanamycin per ml after the specified invasion time.
[b] TNTC, Too numerous to count; ND, not determined.

infected monkeys and guinea pigs (9, 14) and in infection of guinea pig conjunctiva (9, 11, 16). For *Salmonella* (3, 10) and *Shigella* spp. (6, 14), the ability to invade tissue culture cells has been shown to be highly correlated with the ability to produce disease in animal models. The use of cell culture monolayers to evaluate bacterial invasion represents a uniform experimental system under which this complex biological phenomenon can be studied under defined conditions.

In the present study, a method is described that allows for the rapid determination of bacterial invasion. To provide consistent invasion of HeLa cell monolayers, the MOI and the time for bacterial invasion were critical. Increasing the MOI or the invasion time resulted in the recovery of higher numbers of infected HeLa cells. Infection at a low MOI for long periods minimized nonspecific damage to the monolayers as a result of bacterial soluble products, but caused the spreading of bacteria from infected cells during the overlaying procedure.

Bacterial invasion with a high MOI for short invasion periods caused only minimal alterations in cell membranes and produced bacterial colonies which invariably arose from HeLa cell ghosts. For our studies, an invasion time of 90 min with a variable MOI depending on the invasiveness of the bacterial strain maximized recovery with little damage to the monolayer. By using a 90-min invasion period, the efficiency by which a strain can invade HeLa cell monolayers can be determined by the MOI required to give countable wells after overlaying. Therefore, this method provides a convenient method to assess the relative invasiveness of bacterial strains and to examine parameters which modulate the efficiency by which a bacterial pathogen penetrates epithelial cells. Furthermore, bacterial invasion could be observed with as little as 5 min of bacterial contact with the monolayer before washing when a high MOI was used (data not shown). Although bacterial contact may be prolonged during the washing procedure, kanamycin has been shown to rapidly destroy the invasive ability of *S. flexneri* (5). The ability to detect bacterial invasion after very short invasion times is in contrast to results of the studies of Giannella et al. (4) and Ogawa et al. (14, 15), who used invasion times greater than 5 h. Since these studies involved microscopic detection of intracellular bacteria, low levels of infection may have been overlooked.

The use of antibiotics to remove noninternalized bacteria ensured that only infected cells were enumerated. The recovery of internalized bacteria was not reduced over the range of kanamycin concentrations tested, and noninternalized bacteria were removed with a 4-h counterselection. In this regard, washing of the infected monolayers in itself represents an efficient means of reducing or eliminating noninternalized bacteria (Table 1). Additionally, the presence of kanamycin prevents multiple rounds of bacterial infection. Hale and Bonventre (5) have shown that exposure to low levels of kanamycin rapidly destroys the ability of *S. flexneri* to penetrate epithelial cells through the loss of essential metabolic functions. Gentamicin (20 μg/ml) and penicillin-streptomycin (25 U/ml; 25 μg/ml) provide applicable alternatives for counterselection (data not shown). The ability to use alternative antibiotics is important, as many clinical isolates are resistant to one or more antibiotics. As an alternative to antibiotic counterselection, the UV-inactivated bacteriophage lysis procedure of Shaw et al. (22) would appear to be compatible with this method.

We believe that agarose-agar overlays provide a useful tool for studying the complex interactions between an invasive bacteria and a mammalian cell monolayer. This procedure is simple and efficient and represents a substantial improvement over microscopic methods of detection. Furthermore, the method is applicable to a wide range of invasive pathogens and antimicrobial agents. Therefore, this technique should be broadly applicable in the quantitative and qualitative aspects of microbial invasion of mammalian cells.

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