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Plaque Assay for Virulent *Legionella pneumophila*†

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Methods of assessing virulence of *Legionella pneumophila*, the etiologic agent of Legionnaires disease, include the infection of guinea pigs, fertile chicken eggs, and mammalian and protozoan cell cultures. Guinea pig assays, in particular, are expensive, laborious, or unsuitable for routine screening of *Legionella* isolates. We have developed a virulence assay that requires the enumeration of viruslike plaques which are the result of virulent *L. pneumophila* infecting mouse L929 cells. Each plaque is the consequence of the initial infection of an L cell with a single bacterium. A nonvirulent mutant derived from the serial passage of virulent *L. pneumophila* on Mueller-Hinton agar fails to survive within L cells and consequently fails to produce plaques.

Although *Legionella pneumophila*, the etiologic agent of Legionnaires disease (14), is ubiquitous in water, studies have shown that various environmental isolates differed in virulence. Isolates of *L. pneumophila* from hospital potable water have been associated with differences in attack rates in patients (18), in 50% lethal doses and 50% infective doses in guinea pigs (1), and in the ability to survive the killing effects of serum (17). Furthermore, virulence of clinical isolates has been lost by cultivation on bacteriological media (6, 13) or has been regained in laboratory animals and cells in vitro (6, 19). However, a recent study (2) has suggested that the conversion of *L. pneumophila* from virulent to avirulent forms after prolonged cultivation on supplemented Mueller-Hinton agar is a one-way phenomenon.

In assessing the virulence of test strains, it is conventional to use guinea pig assays to determine the 50% lethal dose (1, 5, 6, 7, 13, 14), but these are expensive, time consuming, and often not feasible in a hospital diagnostic laboratory setting. Comparisons of virulence of *Legionella* strains also have been made by using fertile chicken eggs (3, 6, 13), monocyte-macrophages (8, 10), and *Tetrahymena pyriformis* (7). However, these systems do not easily lend themselves to the routine screening of large numbers of strains. We have developed an in vitro virulence assay that is comparatively rapid, relatively inexpensive, and easily amenable to routine testing. This assay utilizes the development and detection of PFU after 4 days of infection of mouse L929 cells with *L. pneumophila*.

MATERIALS AND METHODS

Organisms. A clinical isolate (2064) from the sputum of a patient with Legionnaires disease was used for the virulence studies. This strain was serologically identified as *L. pneumophila* serogroup 1 (OLDA). It was passaged twice on buffered charcoal-yeast extract (BCYE) agar and stored at −70°C in 20% glycerol. For experimental purposes, strain 2064 was serially passaged five times on Mueller-Hinton agar supplemented with hemoglobin and IsoVitaleX (BBL Microbiology Systems, Cockeysville, Md.) (3), at which time a colony (designated 2064M) was picked and a stock culture was prepared on BCYE agar. This was stored at −70°C as described above. When needed, the organisms were grown for 2 days on BCYE agar at 37°C in a humidified CO_2 incubator.

Cell lines. Mouse L929 cells (ATCC clone CCLI) were grown and maintained in minimum essential medium supplemented with 10% heat-inactivated fetal bovine serum (Flow Laboratories, Mississauga, Ontario, Canada), 100 U of penicillin per ml, and 50 μg of streptomycin per ml.

Human A549 cells (lung carcinoma cells obtained from F. Jay, Department of Medical Microbiology, University of Manitoba, Winnipeg, Canada) were grown and maintained under similar conditions but with RPMI 1640 medium.

Virulence determination. Unless otherwise indicated, 10-fold dilutions of 2064 and 2064M ranging from 10^9 to 10^3 organisms per ml were used to inoculate tissue culture cells, fertile chicken eggs, and guinea pigs as described below. In every case, further dilutions were made, and aliquots of each dilution were plated on BCYE agar to determine the initial size of the inoculum.

(i) Cell culture. A549 or L929 cells were seeded in 96-well plates (Costar, Cambridge, Mass.) at a concentration of 10^5 cells per well in 0.1 ml of culture medium (CM) consisting of antibiotic-free RPMI 1640 or minimum essential medium, respectively, with added 1% fetal bovine serum. After 3 h of incubation, duplicate monolayers were inoculated with 2064 or 2064M in 0.1 ml of CM. Three days later the monolayers were examined under an inverted microscope for any discernable cytopathic effect. They were then stained for 2 h at 37°C with 0.01% neutral red (50 μl per well), a dye ordinarily taken up only by viable cells (12). Neutral red-stained cells were washed twice in phosphate-buffered saline, and the dye was then eluted into the well with 100 μl of a 1:1 solution of ethanol and 0.1 M NaH_2PO_4 (12). The color in the plates was subsequently assayed in a Microelisa reader at 570 nm, and the resulting optical density readings were plotted against the number of organisms. From this, the number of organisms causing a 50% reduction of neutral red uptake by control (uninfected) cells was determined.

(ii) Embryonated eggs. Yolk sacs of 8-day-old embryonated White Leghorn hen eggs were infected (6 eggs per dilution) with 0.2 ml of the organisms (14). The eggs were candled daily for 8 days to determine the number of embryos killed. The 50% egg infectivity dose was calculated according to the method of Karber (11).

(iii) Guinea pigs. Male Hartley guinea pigs (High Oak

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† We dedicate this paper to the memory of C. E. van Rooyen, former Head, Department of Microbiology, and Professor Emeritus.

TABLE 1. Comparison of virulence of *L. pneumophila* strains 2064 and 2064M in cell culture, eggs, and guinea pigs

L. pneumophila strain	CFU/ml		
	Cell culture, LD_{50}[a]	Eggs, EID_{50}[b]	Guinea pigs, LD_{50}[c]
2064	3×10^5	1×10^6	1×10^6
2064M	$>6 \times 10^8$	1×10^8	$>1 \times 10^9$

[a] The number of organisms per milliliter causing a 50% reduction in neutral red uptake compared with uptake by uninfected A549 cells. LD_{50}, 50% Lethal dose.

[b] Assayed by the inoculation of yolk sacs of 8-day-old chicken eggs. EID_{50}, 50% Egg infectivity dose.

[c] Assayed by intraperitoneal inoculation of male guinea pigs weighing 250 to 300 g. LD_{50}, 50% Lethal dose.

Ranch, Goodwood, Ontario, Canada), each weighing 250 to 300 g, were infected intraperitoneally (4 animals per dilution) with 1 ml of the organism in saline (7), and the 50% lethal dose was determined as described above.

Plaque assay. Mouse L929 cells were plated in 24-well plates (Costar, Cambridge, Mass.) in CM at a concentration of 5×10^5 per well. After overnight incubation, or alternatively, after allowing the cells to adhere for 2 to 3 h, the resulting confluent monolayers were infected in duplicate with, per well, 0.5 ml of the organisms in CM. After a 60-min adsorption period at 37°C, the inoculum was removed by aspiration, and the monolayers were washed three times with CM containing 50 μg of gentamicin sulfate per ml (wash medium) (3, 9). The cultures were then incubated for an additional 60 min in, per well, 1 ml of gentamicin wash medium, after which the monolayers were washed three times with CM alone prior to adding a 1 ml per well overlay of 0.6% agarose in antibiotic-free minimal essential medium containing 1% fetal bovine serum.

The monolayers were incubated for 4 days at 37°C and then fixed with 10% Formalin. The agarose overlay was removed, and the fixed monolayers were stained with 1% crystal violet in 20% ethanol. The resulting plaques were macroscopically enumerated.

Immunofluorescence. Dilutions of the organisms in CM were added to L929 cells in Lab Tek chamber/slides (Can-Lab, Mississauga, Ontario, Canada). After 60 min at 37°C, bacteria were washed and the monolayers were fixed with Formalin and stained by indirect immunofluorescence (5) with a rabbit anti-*Legionella* antibody prepared as previously described (15) and a fluorescein isothiocyanate-conjugated swine anti-rabbit antibody (Dako, Dimension Laboratories, Mississauga, Ontario, Canada). Cultures were observed microscopically by using a Nikon epifluorescence microscope.

RESULTS AND DISCUSSION

Table 1 shows a comparison of the virulence of 2064 and 2064M in guinea pigs, eggs, and tissue culture. On the basis of these assays, 2064M was deemed to be avirulent.

At a multiplicity of infection (MOI) greater than 10, 2064 produced a complete destruction of A549 and L929 cells. At lower MOIs, the cytopathic effect on A549 cells presented as a generalized form of cell destruction, whereas the cytopathic effect produced by 2064 on L929 cells was focal and resembled that of viral plaques (Fig. 1). There was no overt damage to the monolayer infected with 2064M even at an MOI of 1,000. Since gentamicin kills all bacteria unable to penetrate mammalian cells (3, 9), only those bacteria which had successfully infected the L929 cells eventually caused this focal destruction of the L-cell monolayer. Confirming previous reports, it should be noted that *L. pneumophila* does not grow in this cell-free medium (16).

To determine the number of bacteria necessary to produce a single plaque, serial twofold dilutions of organisms at a starting MOI of 0.01 were plated on the L929 cells in a manner comparable to the Dulbecco (4) dose-response curve of virus plaque assays. The resulting number of plaques,

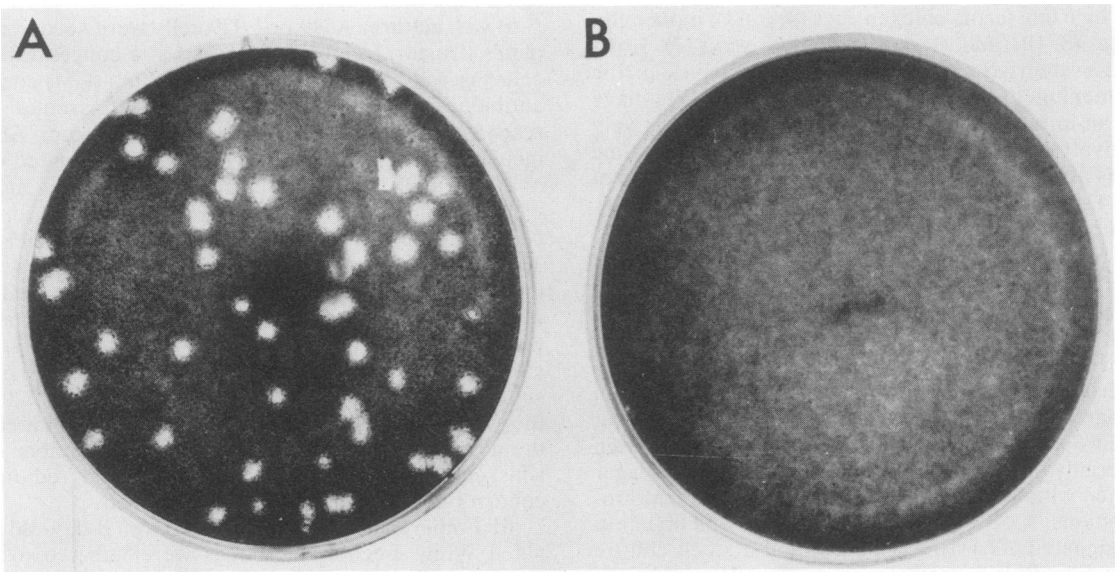

FIG. 1. (A) *L. pneumophila* plaques seen on L929 monolayers. L929 monolayers were infected with *L. pneumophila* for 60 min. The inoculum was removed, washed, and replaced with gentamicin sulfate for an additional 60 min. After the gentamicin was washed away, the monolayers were overlaid with minimal essential medium containing agarose and were incubated for 4 days. The monolayers were then fixed with Formalin; the agarose overlay was removed, and the cells were subsequently stained with crystal violet. (B) Mock-infected L929 monolayers.

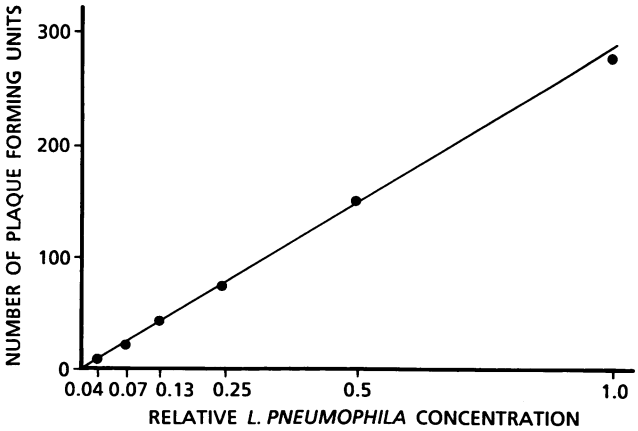

FIG. 2. Plaque formation on L929 monolayers. L929 cells were infected with twofold dilutions of *L. pneumophila*, starting with a concentration of 10^4 organisms per ml as described in the legend to Fig. 1.

when plotted against the dilutions, rendered a linear relationship (Fig. 2), indicating that the initial infection with a single bacterium was sufficient to produce a single plaque.

To investigate whether an enhanced capacity to bind to host cells was related to plaque formation, dilutions of 2064 and 2064M were incubated with L929 cells. Indirect immunofluorescence showed that, at similar concentrations, both strains were equally capable of binding to the L929 cells (Fig. 3). To determine the fate of 2064M after binding, L929 cells were infected with 2064M for 60 min at an MOI of 0.1, according to the method outlined for the plaque assay, except that the agarose overlay was omitted. Instead, 0.5 ml of the culture medium was added to the infected cells. To establish the initial number of 2064M that had penetrated the monolayers, the culture medium was collected and replaced with 0.5 ml of 0.1% Triton X-100 in phosphate-buffered saline (10). After lysis of the monolayers, the lysate was added to the previously collected culture medium and 0.1 ml of this mixture was plated on BCYE agar plates. This gave rise to 500 CFU/ml. The infected monolayers were then lysed as described above at 24, 48, 72, and 96 h postinfection. The resulting number of CFU per ml dropped from 500 to 10 by 24 h and remained as such, indicating that 2064M was rendered nonviable in the L929 cells. Thus, the plaques represent both the ability of the organism to bind and penetrate the host cells and its capacity to survive and multiply intracellularly. The lack of PFU in a *Legionella* plaque assay cannot by itself discriminate between the inability of the organism to either bind or to survive and multiply intracellularly. However, this assay can effectively separate virulent and nonvirulent organisms.

Recently, Dreyfus (3) described an ingestion assay for *L. pneumophila* which used HeLa cells that were infected and overlaid with molten BCYE agar. Organisms which had penetrated the HeLa cells were identified as colonies which grew beneath the BCYE agar.

When we compared our plaque assay on L929 cells with the assay described by Dreyfus on HeLa cells, we observed that serial twofold dilutions yielded 79, 41, and 19 plaques, as opposed to 42, 20, and 9 colonies at an equivalent MOI. Thus, the plaque assay was a more sensitive indicator of virulence by a factor of 2. This appeared to derive from the clarity and ease of enumeration of plaques in L929 cells, as compared with counting the various-sized bacterial colonies which were found randomly on the surface of and beneath the BCYE agar in the Dreyfus assay.

For reasons yet to be determined, the focal nature of the cytopathology identifiable as plaques in L929 cells is unique and quite unlike the generalized destruction caused by *Legionella* infection of Vero, MRC-5, HEp-2, and HeLa cells (16) or A549 cells.

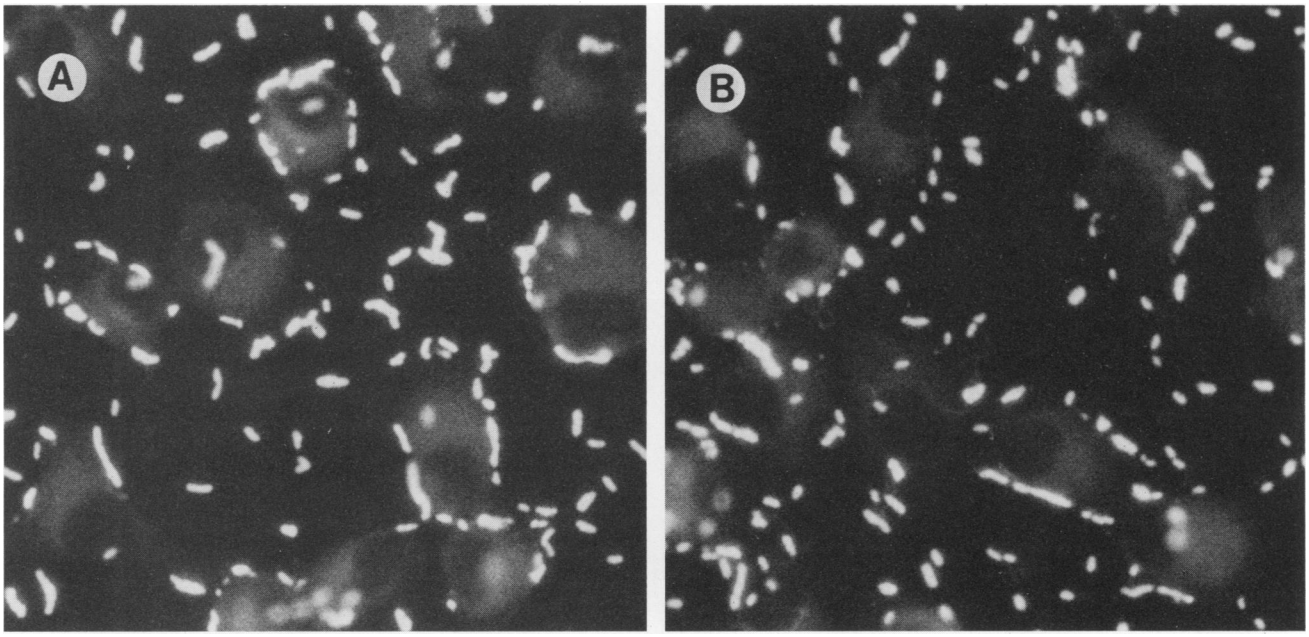

FIG. 3. Immunofluorescence staining of *L. pneumophila* showing the binding of 2064 (A) and 2064M (B) to L929 cells. L929 monolayers were infected with equal numbers (MOI of approximately 100) of 2064 or 2064M for 60 min. After the inocula were removed, the cells were washed, fixed with Formalin, and stained by indirect immunofluorescence.

If virulence may be described as the ability to invade intracellularly, this L929-plaque assay represents a reproducible, simple, yet sensitive assay for virulence in *L. pneumophila*. This phenotypic measure correlates well with other assays of virulence, such as the ability to kill guinea pigs or infect embryonated eggs, but compared with the use of guinea pigs, the plaque assay is much less expensive and less labor intensive. Additionally, while the plaque assay may not completely replace conventional assays, it does provide a means for rapidly screening for virulence, as well as offering a method for the selection and purification of virulent *L. pneumophila*.

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