PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 147, 629-634 (1974)

# Virulence Measurements of *Pseudomonas aeruginosa* Based on Plaque and Toxin Z Formation in Cell Culture[1] (38403)

KURT C. KAMPS AND PETER P. LUDOVICI

*Department of Microbiology and Medical Technology, University of Arizona, Tucson, Arizona 85721*

Many studies have been conducted to correlate various morphological, physical, biochemical, and clinical properties of *Pseudomonas aeruginosa* to $LD_{50}$ determinations in mice (1–3). These attempts to assess strain virulence or pathogenicity have been largely unrewarding because of the lack of strain variability observed with $LD_{50}$ assays.

The present report describes a method of measuring the virulence of clinical strains of *P. aeruginosa* based upon plaque and toxin Z formation in cell culture (4, 5). In this investigation, the efficiency of plaque formation and toxin potency of *P. aeruginosa* strains were correlated to their loss of virulence with subculture. An attempt to assess virulence of the strains *in vivo* using injections of viable organisms in mice ($LD_{50}$ determinations) and using injections of toxin Z in mice (lethality effects) were done as controls.

*Materials and Methods. Cell culture.* HEp-2 cells were cultured in 8 oz glass prescription bottles by methods described previously (4). The medium used was Eagle's basal medium (EBM) with Earle's balanced salt solution (BSS), supplemented with 10% calf serum (CS). A 0.25% trypsin solution in calcium- and magnesium-free phosphate buffer (CMF-PBS) was used to harvest cells.

*Bacteria.* The strains of *P. aeruginosa* employed in the study were clinical isolates obtained from Tucson Medical Center, Tucson, Az. The organisms were maintained on stock agar slants (Difco) and frozen −60° in 1 ml aliquots of 10% CS-EBM using 12 × 75 plastic tubes following 12 hr growth at 37°. After several days in the frozen state two tubes were quick thawed and the bacterial number enumerated by standard plate count methods to obtain an average concentration of the frozen stocks.

*Efficiency of plaquing.* These experiments were conducted by innoculating bacteria into 1 oz glass prescription bottles containing complete HEp-2 cell monolayers. Assay cultures were initiated with an initial inoculum of $10^6$ HEp-2 cells/4 ml in each bottle. After complete monolayer formation, in 24 hr, growth medium was discarded and the cells washed with Dulbecco's phosphate buffered saline (DPBS). One tenth ml of bacterial suspension, containing approximately 100 organisms, was then added and allowed to incubate for 1 hr at 37° to permit adsorption of the bacteria. Four ml of 10% CS-EBM was then added, and bottles were returned to the 37° incubator. Monitoring of plaque formation began 12–14 hr later. When distinct plaques were visible, the cell culture medium was poured off, and, 4 ml of 10% neutral formalin was added for 4 hr to fix the cell monolayer. After fixation, formalin was decanted and monolayers were stained for 5 min with crystal violet. After rinsing of excess dye, and drying a permanent preparation was obtained.

Efficiency of plaquing was determined by dividing the number of plaques observed microscopically by the number of bacteria inoculated (determined by plate count at time of infection of cell culture) and multiplying by 100. Each strain of *P. aeruginosa* was tested in triplicate in three separate trials. Each trial involved fresh cell cultures and separate frozen vials of bacteria.

*Production of toxin Z.* Lethal toxin (toxin Z) of *P. aeruginosa* was prepared as previously described (5). Essentially, this involves inoculation of 100–200 organisms into 8 oz glass pre-

[1] This investigation was supported in part by USPHS Grant AI 10279 and by Biomedical Sciences Support Grant FR 07002 from the General Research Support Branch, Division of Research Resources, Bureau of Health Professions Education and Manpower Training, National Institutes of Health.

scription bottles containing HEp-2 monolayers. The same procedure was followed as described above in the efficiency of plaquing experiments, except here, the cultures were allowed to incubate for 22–24 hr. At this point, the cell culture medium was slightly turbid, the monolayer of cells shows three-quarters cytopathogenicity and the pH of the medium was depressed. Bottles were freeze–thawed twice and the material decanted into 50 ml centrifuge tubes and spun at 10,000*g* for 20 min. The supernatant fluid was then filter sterilized by passage through a Gelman filter-membrane of 0.2 $\mu$m pore diameter. The possible presence of protease in the preparation was assessed by addition of 1 ml of the bacterial free material to 1% skim milk.

In vitro *toxin assay*. If milk was not hydrolyzed, indicating the absence of protease, the potency of Z toxin was measured by the cytopathogenic (CPE) end point procedure using serial dilutions in standard cell culture medium. This test was conducted in 16 × 150 glass tubes containing complete monolayers of HEp-2 cells. The CPE was determined by microscopic examination after 48 hr and recorded as 1+, 2+, 3+, or 4+ representing 25, 50, 75, or 100% destruction of the cell monolayer, respectively. Final end point determination was made 24 hr after decanting the toxin, washing with DPBS, and adding 1 ml of 10% CS-EBM. The end point was the highest dilution in which HEp-2 cells failed to recover from the effect of the toxin.

*$LD_{50}$ determination* in vivo. An attempt to assess the virulence of these *P. aeruginosa* strains was done by injection of previously quantitated suspensions of viable organisms into groups of random bred, 20 g, male and female Swiss Webster mice. The mouse inoculum was prepared by removing a portion of a BHI suspension of the organism to provide $10^{10}$ organisms/ml. This was diluted through $10^4$. Eight mice, four males and four females, were inoculated ip with 0.5 ml of each dilution. The $LD_{50}$ determinations were calculated on mortality rates after 72 hr by the method of Reed and Muench (6).

*Toxin assay* in vivo. Bacterial-free crude toxin Z prepared as previously described, was injected ip into male and female mice. One ml of each toxin was injected into an eight animal group and the mortality noted after 72 hr. No dilutions of these crude toxin preparations was made. Autopsies were performed on animals which died from the effect of Z toxin, and gross pathological changes were noted. (These results will be reported separately.)

*Results. Plaque formation*. The appearance of bacteria plaques in HEp-2 monolayers is illustrated in Fig. 1. The infected cells first round up, swell, and then slowly disintegrate as they pull away from the glass surface. This retraction leaves a homogenous lesion in the cell monolayer which is indistinguishable from a viral plaque. Similar to virus plaques there is an area of focal cell destruction. With most strains of *P. aeruginosa* the bacteria are not easily detectable within the plaques even after fixation and staining of the monolayers. However, with a few strains of *P. aeruginosa* as illustrated in Fig. 2 with strain 140 a thin bacterial colony is visible attached to the glass surface in the center of the plaque.

*Efficiency of plaquing*. Plaque formation produced by adding 100 or fewer organisms to HEp-2 monolayers using a liquid overlay medium resulted in varied efficiencies of plaquing, as illustrated in Table I.

A considerable range of plaque forming efficiency were noted. Strain 029 produced no plaques. This is the first reported strain of *P. aeruginosa* that does not produce plaques in cell culture. Strain 407 initiated plaque formation

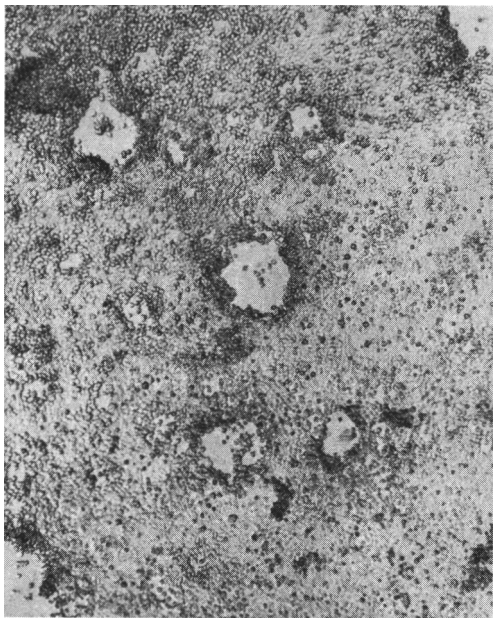

FIG. 1. Plaque formation in HEp-2 monolayer by *P. aeruginosa* strain 321, living unstained culture (×35).

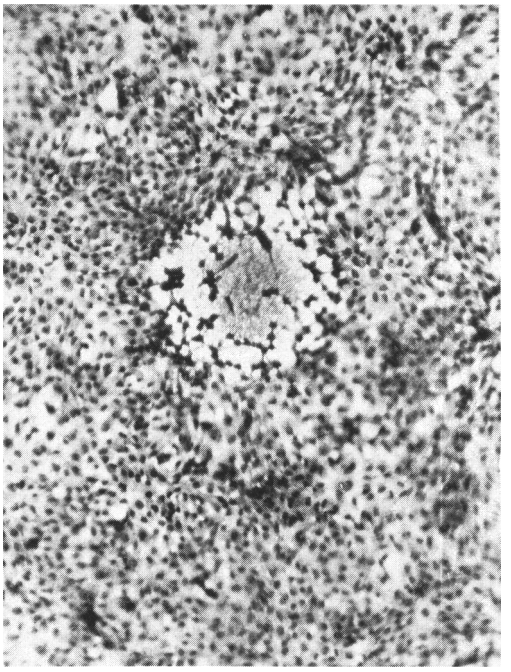

FIG. 2. Plaque formation in HEp-2 monolayer by *P. aeruginosa* strain 140 showing dark staining bacterial colony in plaque center (×60).

only after 18–20 hr which was considerably slower than the 12 hr required by other strains. This may partially account for the low efficiency of plaquing (5%) observed with strain 408. In fact plaques appeared just before the cell monolayer was destroyed by an overgrowth of the organism.

The most important finding, as shown in Table I, was the dramatic intrastrain decrease in efficiency of plaquing observed when the 140 strain was subcultured 10 or 20 times. The efficiency of plaquing decreased from 90 to 26 or 27%. A similar but less dramatic decrease in efficiency of plaque formation occured when strain 321 was subcultured 10 times (55–27%).

A statistical analysis of the data showed that this decrease in the efficiency of plaquing following *in vitro* subculture was significant. An analysis of variance was performed using arcsin transforms of the percent efficiency of plaque formation of each trial with each strain. The means thus obtained, including the confidence limits about those means, are illustrated graphically in Fig. 3. It is readily seen that the substrains 140-10, 140-20 and 321-10 are significantly distinct from their respective parent strains at the 95% confidence level.

Interestingly, interstrain differences in plaquing efficiencies were detected with primary isolated strains but after multiple subculture *in vitro* the differences disappeared.

*Toxin assay. In vitro* potency determinations of the toxins produced by each of the clinical isolates roughly correlated with the efficiency of plaquing data as illustrated in Table II.

A reduction in toxin potency for strain 140 occurred after 10 and 20 subcultures and the loss correlated with the reduction in the efficiency of plaque formation. Strain 321 and its substrain 321-10 did not demonstrate a reduction in toxin potency. This may be simply because the toxin assay is not sufficiently sensitive to detect small differences in toxin potency. Also, the toxin Z employed in these studies was the crude product. Perhaps partial purification and concentration would permit serial twofold dilutions and thus provide more definitive results. Nevertheless, it is of interest to note that there was an overall correlation of toxin potency with efficiency of plaquing. For example, strain 140 was the strongest toxin producer and plaque former while strain 408 was the weakest.

*$LD_{50}$ determinations* in vivo. As previously mentioned, in this procedure Swiss Webster mice were injected ip with tenfold serial dilutions of BHI suspensions of the clinical strains. It is evident from the lethal dose determinations, as shown in Table III, that there was no difference in virulence between any of the strains tested. Thus, no correlation was observed between *in*

TABLE I. Efficiency of Plaque Formation of *P. aeruginosa* Immediately Following Primary Isolation and After Multiple Subculture *in vitro*.

| Isolate no. | Efficiency of plaquing (%) | Range (%) |
|---|---|---|
| 029 | 0[a] | — |
| 029-10 | 0 | — |
| 140 | 90 | 87–100 |
| 140-10 | 26 | 21–32 |
| 140-20 | 27 | 21–38 |
| 321 | 55 | 50–61 |
| 321-10 | 27 | 21–35 |
| 376 | 35 | 21–58 |
| 408 | 5 | 4–7 |

[a] No plaques were formed after repeated attempts with this strain. Growth of the organism in the overlay medium was greatly delayed and no cytopathic effect occurred until gross turbidity existed after 36 hr.

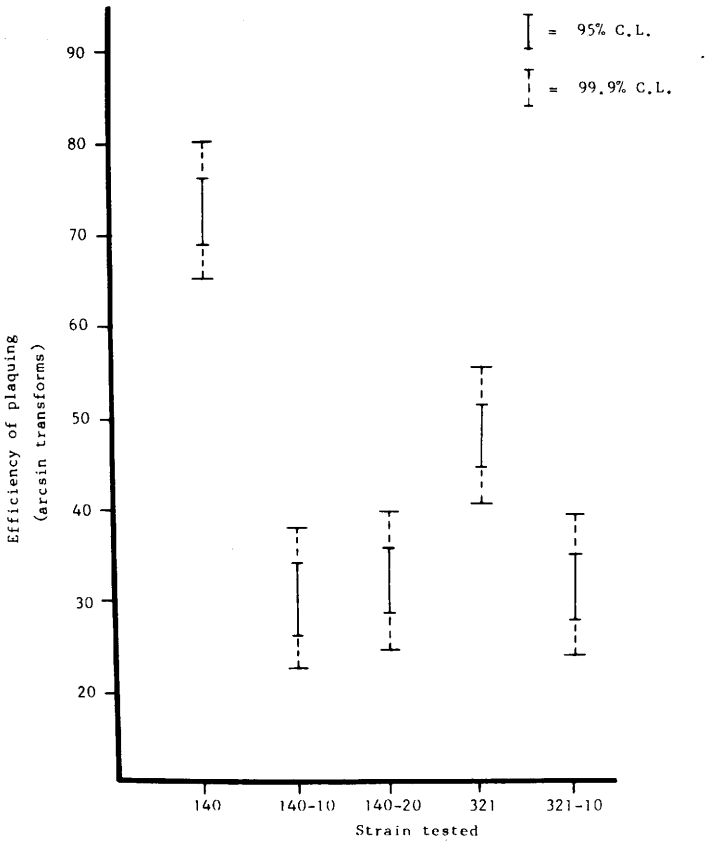


FIG. 3. Confidence limits of efficiency of plaque formation as a measurement of reduced virulence of *P. aeruginosa* with subculture.

*vivo* virulence of the strains for mice and their efficiencies of plaquing or toxin production *in vitro*. This was not an unexpected finding since previous attempts by others to correlate $LD_{50}$ determinations *in vivo* to cultural or other suspected pathogenic properties have also been unsuccessful.

*Toxin potency* in vivo. The percent mortality of mice injected ip with 1 ml of the previously described crude bacterial-free toxin Z preparation is shown in Table IV. Eight animal groups of Swiss Webster mice were employed per strain. These values represent deaths up to 72 hr when the experiment was terminated. A reduction in mortality is observed with subcultures in the case of toxin formed by strains 140-20 but not with strain 140-10. Thus, toxin from strain 140-20 yielded a 25% mortality while toxin from strains 140 and 140-10 yielded a 50% mortality. Similarly, there was 12.5% reduction in mortality with toxins from strains 321 and its substrain 321-10. Toxin Z produced by strain 408 was the least toxic, showing no mortality in mice. This is in agreement with its low cytotoxicity on HEp-2 cells in the *in vitro* potency test (4+ undiluted, Table II).

However, some discrepancies do exist in the data as evidenced by the 50% mortality in toxin from strains 140, 140-10, and 376. These three strains showed variations in their efficiencies of plaquing and *in vitro* toxin potencies. No dilutions of toxin were used in the *in vivo* lethality studies and, thus, here again it is felt that more definitive results would be obtainable following concentration and purification of toxin Z which would permit serial dilution studies.

*Discussion.* Attempts to define and measure virulence of *P. aeruginosa* have involved both studies of the organism itself and its toxic extracellular and intracellular metabolites. Injection of these materials into mice has been the primary test method, with minimal use of cell culture or other mechanisms to aid in evaluation. The primary aim of the research reported here

TABLE II. End Point Titers of Bacterial-Free Toxin Z Produced by Various Isolates of *P. aeruginosa*.

| Isolate no. | CPE[a] | Dilution | CPE | Dilution |
|---|---|---|---|---|
| 140 | 4+ | 1:4 | 3+ | 1:5 |
| 140-10 | 4+ | 1:3 | 3+ | 1:4 |
| 140-20 | 4+ | 1:3 | — | — |
| 321 | 3+ | 1:2 | — | — |
| 321-10 | 3+ | 1:2 | — | — |
| 376 | 4+ | 1:3 | 2+ | 1:4 |
| 408 | 4+ | undilute | 3+ | 1:2 |

[a] CPE graded 1+, 2+, 3+, or 4+ corresponding to 25, 50, 75, and 100% HEp-2 cell destruction, respectively, by the toxin.

was an attempt to assess the virulence of *P. aeruginosa* employing *in vitro* cell culture test conditions. While studies of known toxic enzyme metabolites have served to establish their lethal properties, they have not provided a means of differentiating the virulence of clinical strains. For these reasons, the investigation of a new method was felt justified.

This *in vitro* method, employing plaque and toxin Z production by clinical isolates of *P. aeruginosa* appears to have promise as a virulence assessment procedure. Based upon *in vitro* subculture as a virulence reducing mechanism, Table II indicates the reduced intrastrain efficiency of plaquing obtained by the substrains of strain 140 and 321. Strains 140-10 and 140-20 reflect a 71% lower efficiency of plaquing than their parent strain 140. Similarly, a 51% reduction is observed between parent strain 321 and substrain 321-10. The statistical evidence illustrated in Fig. 3 indicates that efficiency of plaque formation can differentiate the virulence state of a particular strain at the 99.9% confidence level. It is also important to note that the mean values obtained for all the parent strains (Table II) as well as the boundaries indicated in Fig. 3 for strains 140 and 321 indicate that the system is also able to detect interstrain variations in virulence at the time of isolation.

The overlapping confidence limits of strains 140-10, 140-20, and 321-10 in Fig. 3 indicate that after subculture the strains standardized at a low plaquing efficiency which may be a function of the fixed composition of their physiological environment.

The finding (Table III) that there was no difference in the $LD_{50}$ determinations of the tested strains in mice is consistent with the results of other investigators (1–3). The $LD_{50}$ of all the plaque forming strains lies within the log $10^7$. One of the nonplaque forming strains, 029, does present a lower $LD_{50}$. However, this is considered not significant and is attributed to host heterogeneity.

When crude preparations of toxin Z from strain 140 and its substrains 140-10 and 140-20 were applied to cell monolayers, a reduction of CPE was noted in the substrains (Table II). Such a reduction in toxin potency with subculture was not reflected by strains 321 and 321-10. Similarly the percentage mortality of mice injected with undiluted solutions of toxin Z from these strains was not consistent with the effects of toxin Z *in vitro* (compare Table II and IV).

Such inconsistency also was noted when comparing effect of toxin Z *in vitro* by CPE (Table II) with its *in vivo* effect on mouse mortality (Table IV). However, it should be recognized that the preparation employed in these studies was a crude one. When a concentrated and purified toxin Z becomes available in the near future more definitive results will be possible. Nevertheless, the data does show some relationship between the plaquing efficiency of a strain and its toxin producing capacity as measured both *in vitro* and *in vivo*. Also strain 029 could

TABLE III. $LD_{50}$ Determinations in Swiss Webster Mice Injected Intraperitoneally with Clinical Strains and Substrains of *P. aeruginosa*.

| Strain no. | $LD_{50}$[a] |
|---|---|
| 029[b] | $10^{5.895}$ |
| 029-10 | $10^{7.366}$ |
| 140 | $10^{7.428}$ |
| 140-10 | $10^{7-573}$ |
| 140-20 | $10^{7.201}$ |
| 321 | $10^{7.581}$ |
| 321-10 | $10^{7.500}$ |

[a] Calculated by Reed Muench method using eight mice per dilution.

[b] Nonplaque forming strain.

TABLE IV. Percent Mortality of Swiss Webster Mice Injected Interperatoneally with Toxin Z Produced by Clinical Isolates of *P. aeruginosa*.

| Isolate no. | Mortality[a] (%) |
|---|---|
| 140 | 50 |
| 140-10 | 50 |
| 140-20 | 25 |
| 321 | 12.5 |
| 321-10 | 0 |
| 376 | 50 |
| 408 | 0 |

[a] Percentage mortality of eight mice injected intraperitoneally with 1 ml of crude bacterial-free toxin Z.

not produce plaques and was unable to form detectable toxin Z while only 5% of the organisms of strain 408 could produce plaques which correlated well with its low toxin Z concentration as measured *in vitro* and *in vivo* (Tables I, II, and IV). Consequently, this study has provided additional evidence supporting the theory that plaque formation is dependent upon the production of toxin Z (5).

Perhaps the most important finding in this report is that efficiency of plaquing and toxin potency progressively decreased as the organism is farther removed (subcultured) from the original *in vivo* site of infection, simulating reduction in virulence. Conversely, the $LD_{50}$ determinations of these same organisms does not reflect any difference or change as a function of subculture. This latter finding is, as stated, consistent with those obtained by others (1–3).

This fact is the basis for the belief that the plaque forming ability of this organism may be a measure of its virulence. It is at least the first biological system to provide a means of measuring the destructive potential of the organism under simulated host conditions. Certainly the plaque phenomenon *per se*, the focal destruction of living cells, is a virulent manifestation. It has long been established that the various toxic metabolites produced by an organism may be responsible for its virulence. This suggests the possibility that toxin Z may be responsible for plaque formation. The exact mechanism of plaque formation has not been defined but a cell to cell poisoning by toxin Z via cytoplasmic bridges from the initial infected cell does not seem an entirely unreasonable hypothesis. Additional data on toxin Z is now being collected and will be published in the near future. It is not related to any *Pseudomonas aeruginosa* toxin reported in the literature and its lethal effect in mice is uniquely directed to the destruction of liver parenchyma (7).

*Summary.* An *in vitro* system was devised to measure the virulence of *Pseudomonas aeruginosa* based on the organism's ability to form virus-like plaques and toxin Z in cell culture monolayers.

Plaquing efficiency varied among clinical strains and also between the original isolates and their substrains after 10 and 20 subcultures. These variations were significant at the 99.9% confidence level.

The data on the potency of toxin Z produced by these strains as measured by cytopathogenic effect in cell culture and by mouse lethality generally agreed with the data on their plaquing efficiency. Conversely no differences between the tested strains were detected in $LD_{50}$ determinations in mice. This latter result is in agreement with the findings of other investigators and reemphasizes the need for a more reliable indicator system to assess the virulence of *P. aeruginosa*. Plaquing efficiency and toxin Z formation in cell culture appear to hold considerable promise as indicators that may satisfy this need.

1. Gaby, W. L., and Logan, C., J. Bacteriol. **82,** 149 (1961).

2. Klyhn, K. M., and Gorrill, R. H., J. Gen. Microbiol. **47,** 227 (1967).

3. Kobayashi, F., Jap. J. Microbiol. **15,** 295 (1971).

4. Ludovici, P. P., and Christian, R. T., Proc. Soc. Exp. Biol. Med. **131,** 301 (1969).

5. Coleman, R. G., Janssen, R. J., and Ludovici, P. P., Proc. Soc. Exp. Biol. Med. **131,** 311 (1969).

6. Reed, L. J., and Muench, H., Amer. J. Hyg. **27,** 493 (1938).

7. Ludovici, P. P., Kamps, K. C., and Schloss, G. T., In Preparation.

Received April 18, 1974. P.S.E.B.M., 1974, Vol. 147.