# VIRULENCE AND CITRULLINE UREIDASE ACTIVITY OF *PASTEURELLA TULARENSIS*[1, 2]

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ABSTRACT

MARCHETTE, NYVEN J. (University of Utah, Salt Lake City), AND PAUL S. NICHOLES. Virulence and citrulline ureidase activity of *Pasteurella tularensis*. J. Bacteriol. **82**:26–32. 1961.—The presence of a citrulline ureidase system in *Pasteurella tularensis* strains of high virulence, and its absence in avirulent strains and strains of low virulence was confirmed. The presence of this sytem, however, was shown to be not directly related to virulence.

The only wild strain of *P. tularensis* tested that lacked a citrulline ureidase system was isolated from a rodent. All the strains, isolated from rabbits, rabbit ticks, a human being, and a horse, that were tested possessed this system.

The existence of two North American varieties of *P. tularensis* was postulated on the basis of virulence and citrulline ureidase activity.

The presence of citrulline ureidase in highly virulent strains of *Pasteurella tularensis* and its absence in strains of low or no virulence have been reported by Fleming and Foshay (1955). Except for differences in growth rate on artificial media, this is the only qualitative physiological difference between *P. tularensis* strains of different virulence that has been found to date. Although there appears to be a direct correlation between the virulence of a strain and its ability to metabolize citrulline with the formation of ornithine, $CO_2$, and $NH_3$, this has not been investigated in detail.

In the present study, the relationship of citrulline ureidase activity to virulence of *P. tularensis* was tested, and the results used to characterize recent isolates and old laboratory strains.

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[2] This is Ecological and Epizoological Research Laboratory contribution no. 49.

## MATERIALS AND METHODS

*Cultures for virulence determinations.* The 18 strains of *P. tularensis* used are listed in Table 1. All stock cultures were maintained on glucose cysteine blood agar at 4 C, with routine monthly transfers to fresh media.

Suspensions for the inoculation of animals were prepared either from 48-hr aerated cultures in modified casein partial hydrolyzate broth (Mills, 1954), or from a 24- to 48-hr glucose cysteine blood agar culture. The 48-hr broth cultures regularly contained $1 \times 10^9$ to $5 \times 10^9$ viable organisms per ml, as determined by viable counts, using the method recommended by Snyder (1947). The agar cultures, when washed with normal saline (0.85% NaCl) and adjusted to 24% light transmittance on a spectrophotometer set at 525 m$\mu$, regularly contained $1 \times 10^9$ to $5 \times 10^9$ viable organisms per ml. Serial, 10-fold dilutions for viable counts and animal inoculation were routinely prepared in normal saline containing 0.2% gelatin. Plates for viable counts were always inoculated prior to animal inoculation.

*Assay of citrulline ureidase activity.* Cultures of each of the 18 strains of *P. tularensis* were prepared in casein partial hydrolyzate broth or Snyder's peptone broth at pH 6.8. After 48 hr of incubation at 35 to 37 C on a reciprocating shaker, the cultures were checked for purity by microscopic examination, washed twice by centrifugation in normal saline, and suspended in minimal amounts of deionized water. The cell suspensions were then lysed by sonic vibration in a sonic oscillator (50 watt, 9 kc/sec). The cell lysates were kept at −20 C for 1 to 3 weeks before use. Storage of lysates for as long as 6 months had no effect on citrulline ureidase activity.

Total nitrogen was determined for each lysate according to the method of Lang (1958).

TABLE 1. *Strains of Pasteurella tularensis used in this study*

| Strain | Source | Location | Isolated by | Date of isolation | Pertinent reference |
|---|---|---|---|---|---|
| Schu A | Human | U. S. | Foshay | 1941 | Bell, Owen, and Larson, 1955 |
| $Jap_4$ | Human | Japan | Ohara | 1926 | Moody and Downs, 1955 |
| 38 | Human | U. S. | Francis | 1920 | Hesselbrock and Foshay, 1945 |
| $425F_4G$ | *Dermacentor andersoni* | U. S. | RML* | | Bell et al., 1955 |
| Kf-473 | *Microtus montanus* | Oregon | RML | 1957 | Jellison, Bell and Owen, 1959 |
| H-8859 | Horse | Montana | Claus | 1958 | Claus, Newhall, and Mee, 1959 |
| NIIEG-Blue | ? | Russia | ? | ? | Anonymous 1944–1946; Fairbich and Rumarina, 1946 |
| NIIEG-Gray | ? | Russia | ? | ? | Anonymous, 1944–1946; Fairbich and Rumarina, 1946 |
| DPG-1 | *Dermacentor parumapertus* | Utah | Stoenner | 1956 | Stoenner et al., 1959 |
| DPG-2 | *Lepus californicus* | Utah | Stoenner | 1956 | Stoenner et al., 1959 |
| DPG-3 | *D. parumapertus* | Utah | Stoenner | 1956 | Stoenner et al., 1959 |
| DPG-4 | *L. californicus* | Utah | Stoenner | 1956 | Stoenner et al., 1959 |
| DPG-5 | *D. parumapertus* | Utah | Stoenner | 1956 | Stoenner et al., 1959 |
| DPG-6 | *D. parumapertus* | Utah | Stoenner | 1956 | Stoenner et al., 1959 |
| SKV-1 | *Sylvilagus audubonii* | Utah | Ecol. Res.† | 1958 | Unpublished |
| SKV-2 | *L. californicus* | Utah | Ecol. Res. | 1958 | Unpublished |
| SKV-3 | *L. californicus* | Utah | Ecol. Res. | 1959 | Unpublished |
| 9K-161 | Jack rabbit ticks | Utah | Ecol. Res. | 1959 | Unpublished |

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Citrulline ureidase enzyme activity of sonic lysates was assayed according to the procedure described by Fleming and Foshay (1955). The reaction mixture containing 53.8 μmoles DL-citrulline and a sample of sonic lysate containing 1.50 mg bacterial nitrogen was buffered at pH 6.5 with phosphate buffer to a total volume of 1.5 ml and incubated at 30 C. Samples removed at regular intervals were applied to washed Whatman no. 1 filter paper and chromatographed at room temperature in *n*-butanol-pyridine-water (1:1:1) solvent system for 3 to 4 hr, then dried for 20 min at 65 C, and finally sprayed with 0.5% alkaline solution of ninhydrin in 71% ethyl alcohol and developed at 65 C for 22 min. The ornithine spots were eluted with 71% alcohol and the optical density of the solution determined at 575 mμ in a spectrophotometer (Kay, Harris, and Entenman, 1956).

The amount of ornithine produced was calculated by referring to a previously determined standard curve relating micromoles of ornithine to optical density of the eluates.

*Virulence titrations.* The virulence of each strain of *P. tularensis* was determined by titration in deer mice (*Peromyscus maniculatus*), guinea pigs (Hartley strain), and white rabbits. The deer mice were laboratory reared and were 4 to 8 generations removed from the original wild parent stock.

For each titration, 30 to 60 deer mice, 4 to 40 guinea pigs, and 2 to 6 rabbits were used. The number of deaths from tularemia occurring over a 21-day period was recorded and the $LD_{50}$ of each strain determined by probit analysis (Finney, 1952). In some cases laboratory reared wood rats (*Neotoma lepida*) and grasshopper mice (*Onychomys leucogaster*) also were used (Egoscue, 1957, 1960).

## RESULTS

*Virulence of P. tularensis.* The virulence of 12 recently isolated strains of *P. tularensis* was compared with 6 laboratory strains (Table 2). For purposes of this study, a virulent strain is defined as one for which the $LD_{50}$ for mice, guinea pigs, and rabbits is 1 to 10 organisms inoculated subcutaneously; a moderately virulent strain has

TABLE 2. *Virulence and citrulline ureidase activity of six laboratory and twelve wild strains of Pasteurella tularensis*

| Strain | Subcutaneous $LD_{50}$ | | | Ornithine produced per mg bacterial N* |
|---|---|---|---|---|
| | Deer mice | Guinea pigs | Rabbits | |
| | | | | *μmoles* |
| *Laboratory strains:* | | | | |
| Schu A | <10 | <10 | <10 | 24.5 |
| $Jap_4$ | $7.4 \times 10^6$ | $>10^8$ | $>10^9$ | 0.0 |
| 38 | $>10^9$ | $>10^9$ | $>10^9$ | 0.0 |
| NIIEG-Blue | $1.2 \times 10^3$ | | $>10^9$ | 0.0 |
| NIIEG-Gray | $>10^9$ | $>10^9$ | $>10^9$ | 0.0 |
| $425F_4G$ | <10 | $>10^4$ | $>10^9$ | 0.0 |
| *Wild strains:* | | | | |
| Kf-473 | <10 | <10 | $>10^4$ | 0.0 |
| H-8859 | <10 | <10 | <10 | 20.0 |
| DPG-1 | <10 | <10 | <10 | 4.8 |
| DPG-2 | <10 | | | 4.5 |
| DPG-3 | <10 | | | 4.5 |
| DPG-4 | <10 | <10 | <10 | 4.8 |
| DPG-5 | <10 | <10 | <10 | 4.5 |
| DPG-6 | <10 | <10 | <10 | 6.0 |
| SKV-1 | <10 | <10 | <10 | 7.5 |
| SKV-2 | <10 | | | 8.0 |
| SKV-3 | <10 | | | 4.0 |
| 9K-161 | <10 | <10 | <10 | 6.0 |

* Reaction mixture contained 53.8 μmoles of DL-citrulline and was incubated at 30 C for 6 hr.

an $LD_{50}$ for mice of 1 to 10, for guinea pigs $10^4$ or lower, and for rabbits greater than $10^4$ organisms; a low virulent strain has an $LD_{50}$ for mice of greater than $10^3$ organisms and is avirulent for guinea pigs and rabbits. Avirulent strains do not kill mice, guinea pigs, or rabbits.

The Schu A strain and all the recent isolates, except strain Kf-473, are virulent; strain Kf-473 and $425F_4G$ are moderately virulent; $Jap_4$ and NIIEG (blue var.) are of low virulence; NIIEG (gray var.) and 38 are avirulent. Only virulent strains, as defined above, possessed citrulline ureidase activity (Table 2).

*Quantitative difference in citrulline ureidase activity.* The enzyme activity in lysates of each strain was estimated by determining the amount of ornithine produced from a standard amount of citrulline per mg of bacterial nitrogen in the lysate. The results of these determinations are shown in Table 2. Strains Schu A and H-8859 showed the greatest activity, reaching equilibrium after approximately 2 to 4 hr of incubation at 30 C. The activity of all the DPG, SKV, and 9K-161 strains was very low. Equilibrium was not reached until 48 hr or longer. The activity in the latter ten strains was nearly identical.

The difference in citrulline ureidase activity among the active strains was further investigated by comparing the activity of different concentrations of Schu A lysates with the activity of the standard lysate of strain SKV-1. Schu lysates containing 0.24 mg N were about comparable to lysates of the less active SKV-1 strain containing 1.50 mg N (Fig. 1). This is approximately a 6-fold difference in activity.

To test the effect of change in virulence on citrulline ureidase activity, attempts were made to enhance the virulence of the low virulent $Jap_4$ strain by serial passage through resistant deer mice and through susceptible grasshopper mice. The parent strain is avirulent for wood rats and the subcutaneous $LD_{50}$ for deer mice is $7.4 \times 10^6$, and in grasshopper mice $1.2 \times 10^1$ organisms (Marchette et al., 1961). Heart blood from deer mice or grasshopper mice dead or dying of tularemia was inoculated intraperitoneally into healthy deer mice or grasshopper mice, respectively. At the end of six such serial passages in grasshopper mice, the organism was isolated from heart blood and tested for virulence for deer mice. Doses as high as $1.2 \times 10^4$ viable organisms inoculated subcutaneously failed to kill any deer mice.

After 15 serial passages, a culture was isolated from each species and its virulence for deer mice again tested. All deer mice receiving one or more viable organisms from either culture died of tularemia within 12 days after inoculation. Thus, the virulence for deer mice had increased a million-fold.

Each strain was then serially passed an additional 15 times through each species of mouse via the subcutaneous route, after which the virulence of each was titrated in deer mice and wood rats. One organism of each strain was capable of killing deer mice. Most wood rats survived doses as high as $10^7$ viable organisms inoculated subcutaneously, but a few died at doses as low as $10^5$ (Table 3). This indicates that the virulence for deer mice and wood rats had increased over that of the parent strain. Neither of these strains, however, had developed the ability to metabolize citrulline.

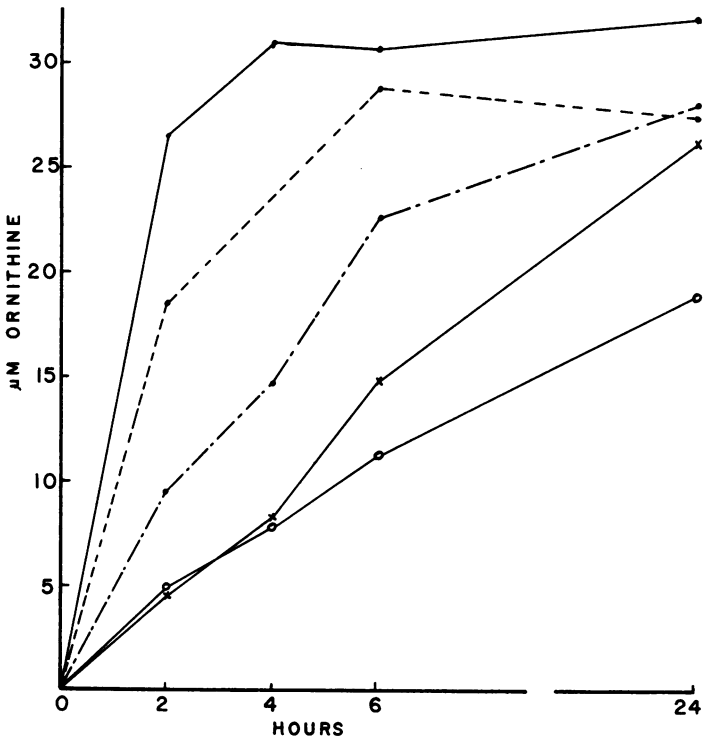


FIG. 1. *Ornithine production from a standard amount of citrulline (52 μmoles) as a function of nitrogen concentration of Schu A cell lysate compared to strain SKV-1 with a nitrogen concentration of 150 mg. This strain is taken as representative of all the SKV, DPG, and 9K-161 strains.* (●——●) Schu 1.92 mg N; (●-----●) *Schu 0.96 mg N;* (×——×) *Schu 0.48 mg N;* (●—·—●) *Schu 0.24 mg N;* (○——○) *SKV-1 1.50 mg N.*

TABLE 3. *Virulence for wood rats of Pasteurella tularensis strain Jap$_4$ (parent), Jap$_4$-Pm-30 (30 serial passages in deer mice), and Jap$_4$-01-30 (30 serial passages in grasshopper mice)*

| Jap$_4$ substrain | Number of organisms | | | | | |
|---|---|---|---|---|---|---|
| | $10^8$ | $10^7$ | $10^6$ | $10^5$ | $10^4$ | $10^3$ |
| Parent | 0/8* | 0/8 | 0/8 | 0/8 | 0/8 | 0/8 |
| Pm-30 | | 2/4 | 1/4 | 1/4 | | |
| 01-30 | | 2/8 | 0/8 | 0/5 | 0/7 | 0/8 |

* Data shown as the ratio of the number of animals dying of tularemia over a 21-day period to the total number used.

Since no drastic change in virulence or citrulline ureidase activity was accomplished by animal passage, an attempt was made to induce variation directly. Eigelsbach, Braun, and Herring (1951) were able to produce a high percentage of variants of the Schu A strain by utilizing the method of Stakman et al. (1948). Peptone broth (pH 6.8) containing 0.01% uranium acetate was inoculated with a heavy suspension of *P. tularensis* Schu A cells and incubated at 37 C without aeration for 15 days. Samples were then subcultured on peptone agar plates (pH 7.2). The isolated colonies obtained were observed under oblique light (Henry, 1933), and under direct illumination after staining with 1:1,000 solution of crystal violet (White and Wilson, 1951). Approximately 90% of the colonies were translucent, bluish colored under oblique light illumination, stained a deep violet-red with crystal violet, and were nonsmooth in appearance. The remainder of the colonies were smooth, opaque, and whitish colored under oblique light, and stained blue with crystal violet. Each colony form was isolated in pure culture and subcultured for several passages on peptone agar or glucose cysteine blood agar without reverting to the parent type.

Each variant was cultured in Snyder's peptone

TABLE 4. *Virulence of smooth (S) and nonsmooth (NS) variants of Pasteurella tularensis strain Schu A for deer mice and rabbits*

| Variant of Schu A | Number of organisms* | | | |
|---|---|---|---|---|
| | $10^7$ | $10^5$ | $10^3$ | $10^1$ |
| *Smooth* | | | | |
| Deer mice | | 4/8 | 3/8 | 2/8 |
| Rabbits | 0/2† | 0/2 | 0/2 | |
| *Nonsmooth* | | | | |
| Deer mice | | 0/8 | 0/8 | 0/8 |
| Rabbits | 0/2 | 0/3 | 0/3 | |

* Number of organisms shown ×3 for the smooth variant; number of organisms shown ×10 for the nonsmooth variant.

† Data shown as the ratio of the number of animals dying of tularemia, in a 21-day period after subcutaneous inoculation, to the total number used.

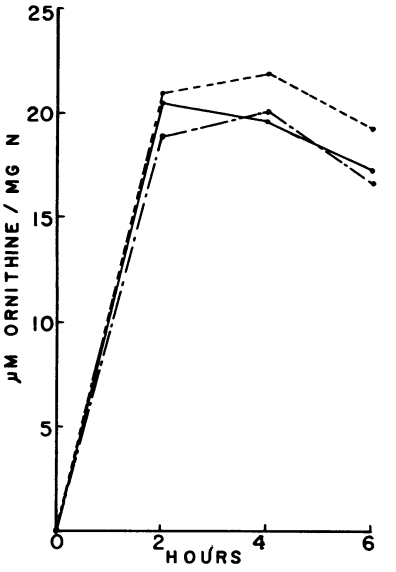


FIG. 2. *Citrulline ureidase activity of smooth (S) and nonsmooth (NS) variants of Pasteurella tularensis strain Schu A, compared to that of the parent strain.* (——) *Schu (parent);* (-----) *Schu (S);* (—·—) *Schu (NS).*

broth (pH 6.8) for 48 hr at 35 to 37 C on a reciprocal shaker. A sample of each was removed for virulence titrations in deer mice and rabbits. The remainder was washed and lysed according to the standard procedures described, and tested for citrulline ureidase activity.

The nonsmooth (NS) variant failed to kill any deer mice in doses up to $4 \times 10^5$ viable organisms. Rabbits survived doses as high as $1 \times 10^8$ organisms. The smooth (S) variant was partially virulent for deer mice, but avirulent for rabbits (Table 4). The citrulline ureidase activity of both smooth and nonsmooth variants, however, had not decreased and was the same as that of the parent strain (Fig. 2).

## DISCUSSION

The only naturally occurring strains of *P. tularensis* tested that possessed the ability to metabolize citrulline were those virulent for mice, guinea pigs, and rabbits. None of the strains that are avirulent or of attenuated virulence possessed any demonstrable citrulline ureidase activity.

Attempts to increase the virulence of the low virulent $Jap_4$ strain by serial passage through a susceptible and a resistant host, respectively, were only partially successful. Strains virulent for deer mice were obtained but the virulence for wood rats, which are completely resistant to infection with the parent strain, was only slightly altered. This slight increase in virulence was not accompanied by development of citrulline ureidase activity. In addition, it was possible to select low virulent variants of the virulent Schu strain which retained the ability to break down citrulline. Thus, no direct relationship between virulence and citrulline ureidase activity in *P. tularensis* could be demonstrated.

There was a difference in the citrulline ureidase activity in the rabbit and rabbit tick strains of *P. tularensis* compared to the human (Schu) and the horse (H-8859) strains. These strains are all equally virulent for mice, guinea pigs, and rabbits, yet the rabbit strains possessed only about one-sixth the citrulline ureidase activity per unit of bacterial nitrogen of the Schu and H-8859 strains.

The results suggest that the presence or absence of the citrulline ureidase system (and possibly its level of activity) and virulence are characteristics which may define two or possibly three varieties of *P. tularensis*: (i) virulent strains possessing citrulline ureidase enzyme activity of a high order (Schu A and H-8859); (ii) virulent strains with a low order of activity (DPG, SKV, and 9K-161); and (iii) strains of lower virulence lacking this system entirely (Kf-473, $425F_4G$, $Jap_4$, 38, and NIIEG).

The non-citrulline ureidase-possessing strain

(Kf-473) was isolated from a rodent (*Microtus montanus*). All the strains possessing citrulline ureidase activity of a low order were isolated from rabbits or rabbit ticks (*Dermacentor parumapertus*). The only two strains possessing enzyme activity of a high order, H-8859 and Schu A, were isolated from a horse and a man, respectively. Since the horse and human being are probably only accidental hosts of *P. tularensis*, and play no significant role in the ecology of this agent, it is possible that the two strains are actually derived from rabbit strains. If this is the case, then only two wild varieties are probably present in nature—the lower virulent rodent strains, lacking this system; and the virulent rabbit strains, possessing it.

Jellison et al. (1960) have arrived independently at the same conclusion, using different criteria. On the basis of reservoir host, virulence, seasonal distribution, and geographical occurrence, they suggest that there are two major kinds of tularemia in North America, one a tick-borne tularemia of rabbits, and the other, a water-borne tularemia of rodents.

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## LITERATURE CITED

Anonymous. 1944–1946. Tularemia. Excerpts from the published literature on the development of NIIEG varieties. Tico-5954 55-INT-397, 1936–1946. Trans. No. 41, 7 p.

Bell, J. F., C. R. Owen, and C. L. Larson. 1955. Virulence of *Bacterium tularense*. I. A study of the virulence of *B. tularense* in mice, guinea pigs, and rabbits. J. Infectious Diseases **97**: 162–166.

Claus, K. D., J. H. Newhall, and D. Mee. 1959. Isolation of *Pasteurella tularensis* from foals. J. Bacteriol. **78**:294–295.

Egoscue, H. J. 1957. The desert wood rat. A laboratory colony. J. Mammal. **38**:472–481.

Egoscue, H. J. 1960. Laboratory and field studies of the northern grasshopper mouse. J. Mammal. **41**:99–110.

Eigelsbach, H. T., W. Braun, and R. Herring. 1951. Studies on the variation of *Bacterium tularense*. J. Bacteriol. **61**: 557–570.

Fairbich, M. M., and T. S. Rumarina. 1946. Dry living tularemia vaccine of NIIEG of Red Army. Zhur. Microbiol. Epidemiol. Immunobiol. **7**:59–63; **10**:23–27.

Finney, D. J. 1952. Probit analysis. Cambridge Univ. Press, London. 318 p.

Fleming, D. E., and L. Foshay. 1955. Studies on the physiology of virulence of *Pasteurella tularensis*. I. Citrulline ureidase and deamidase activity. J. Bacteriol. **70**:345–349.

Henry, B. S. 1933. Dissociation in the genus *Brucella*. J. Infectious Diseases **52**:374–402.

Hesselbrock, W., and L. Foshay. 1945. The morphology of *Bacterium tularense*. J. Bacteriol. **49**:209–231.

Jellison, W. L., J. F. Bell, and C. R. Owen. 1959. Mouse disease studies. The Oregon meadow mouse irruption of 1957–1958, p. 71–80. Fed. Coop. Ext. Serv., Oregon State College, Corvallis.

Jellison, W. L., C. R. Owen, J. F. Bell, and G. Kohls. 1960. Tularemia and animal population. Ecology and epizoology. Paper presented at Annual Mid-west Wildlife Conference, Toronto, Canada. Dec. 6, 1960.

Kay R. E., D. C. Harris, and C. Entenman. 1956. Quantification of the ninhydrin color reaction as applied to paper chromatography. Arch. Biochem. Biophys. **63**:14–25.

Lang, C. A. 1958. Simple microdetermination of Kjeldahl nitrogen in biological materials. Anal. Chem. **30**:1692–1694.

Marchette, N. J., D. L. Lundgren, P. S. Nicholes, and E. D. Vest. 1961. Studies on infectious diseases in wild animals in Utah. I. Susceptibility of wild animals to experimental tularemia. Zoonoses Research **1**(3): 49–73.

Mills, R. C. 1954. Research studies on the growth and metabolism of *Bacterium tularensis*. Univ. Kans. Quart. Prog. Rept. of Research to the U. S. Army Chemical Corps. 1 Jan.–31 Mar., 1954.

Moody, M. D., and C. M. Downs. 1955. Studies on tularemia. I. The relation between certain pathogenic and immunogenic properties of variants of *Pasteurella tularensis*. J. Bacteriol. **70**:297–304.

SNYDER, T. L. 1947. The relative errors of bacteriological plate counting methods. J. Bacteriol. **54**:641–654.

STAKMAN, E. C., J. M. DALEY, M. L. GATTANI, AND I. WAHL. 1948. Variation induced by uranium nitrate in corn smut and the cultivated mushroom. Science **108**:554.

STOENNER, H. G., R. HOLDENRIED, D. LACKMAN, AND J. S. ORSBORN, JR. 1959. The occurrence of *Coxiella burneti*, *Brucella* and other pathogens among the fauna of the Great Salt Lake Desert in Utah. Am. J. Trop. Med. Hyg. **8**:590–596.

WHITE, P. G., AND J. B. WILSON. 1951. Differentiation of smooth and nonsmooth colonies of *Brucella*. J. Bacteriol. **61**:239–240.