APPLIED MICROBIOLOGY, May 1971, p. 809-814
Vol. 21, No. 5

Application of Immunofluorescence to Studies on the Ecology of *Sphaerophorus necrophorus*

M. M. GARCIA, D. H. NEIL, AND K. A. McKAY
Animal Pathology Division, Canada Department of Agriculture, Hull, Quebec, Canada

Received for publication 22 September 1970

Sphaerophorus necrophorus (bovine liver abscess isolates) antiserum was fractionated and labeled with fluorescein isothiocyanate. The fluorescent-antibody (FA) conjugate proved to be species-specific and facilitated the detection of *S. necrophorus* cells in liver abscesses, viscera, and ruminal contents of cattle. Brightly fluorescing *S. necrophorus* cells were observed in experimentally inoculated soil incubated anaerobically at 37 and 4 C for 8 and 10 months, respectively. When incubated under moist conditions (80% water holding capacity) at 37 C, the test organism survived in both sterile and unsterile soil for as long as 8 weeks. Results strongly support the feasibility of using FA techniques concurrent with cultural methods for rapid detection of *S. necrophorus* infections.

Sphaerophorus necrophorus is a highly pleomorphic, gram-negative, obligate anaerobe which can produce necrotic, gangrenous, or purulent lesions in man and animals (2, 7, 8). Its ability to induce hepatic abscesses in cattle has caused considerable economic loss through the condemnation of infected livers (15). Moreover, its consistent association with enzootic foot-rot infections in cattle and sheep has led to the growing suspicion that this pathogen could survive in soil (10, 12). Knowledge of the ecology of this pathogen is meager despite its apparent widespread distribution probably because convenient methods for its detection are not available.

This study was undertaken to explore the feasibility of applying immunofluorescence, concurrent with cultural methods, to investigate certain aspects of the ecology and survival of *S. necrophorus*.

MATERIALS AND METHODS

Organism and media. Ten bovine liver abscesses were obtained from freshly slaughtered animals and brought to the laboratory for study. *S. necrophorus* was isolated from each abscess by the decimal dilution technique of Calkins and Schrivner (5). The isolates were grown in fluid thioglycolate medium and cooked meat broth or streaked on blood-agar plates (5% defibrinated bovine blood) and incubated at 37 C under a 5% CO_2 plus 95% N_2 or H_2 gas phase. Identification of *S. necrophorus* isolates was verified by morphological, cultural, pathogenicity, and antibiotic sensitivity tests (4, 8, 9). For inoculum and antigen preparations, 24- to 48-hr cultures were used. In each case, retrospective checks of the purity of the culture were made on 5% bovine blood-agar.

Preparation of conjugated antiserum. Washed *S. necrophorus* cells were suspended in 0.3% formolized phosphate-buffered saline (PBS, *p*H 7.1) and adjusted to a turbidity equivalent to that of Brown's opacity tube no. 8 (Burroughs Wellcome and Co., London). Since there were no detectable variations among the 10 *S. necrophorus* isolates, their growth was pooled into three batches of antigen. Young rabbits (four per batch of antigen and one control) were bled before injection. Preimmune serum was used as a control and for verifying the absence of antibodies against *S. necrophorus*. An initial dose of 0.5 ml of a 1:1 antigen-sodium alginate (Colab, Weston, Ontario) mixture was injected subcutaneously into four sites of each rabbit's abdomen. This was followed by 10 intravenous injections spaced every other day for 4 weeks with doses increasing from 0.1 to 2.0 ml. Final bleeding was done after a 10-day pause from the last injection.

Antibodies against *S. necrophorus* were detected by agglutination and diffusion techniques. The latter technique was also applied to both intact cells and crude cell fractions. Cells were broken by ultrasonic disruption for 10 min. Crude cell wall fraction was sedimented after spinning the disrupted cells at 18,000 $\times$ *g* for 10 min. The 18,000 $\times$ *g* supernatant constituted the cytoplasmic fraction.

Antiserum was fractionated by two methods, the first involving direct ammonium sulfate precipitation (14) and the second procedure employing rivanol (2-ethoxy-6,9-diaminacridine lactate) to obtain a chromatographically pure gamma globulin in solution (1). The gamma globulins obtained by both fractionation methods were conjugated with fluorescein isothiocyanate (FITC; reference 14).

Acetone-fixed smears were stained with fluorescent antibody (FA) for 30 min in a moist chamber, washed with two changes of PBS, and mounted in buffered

glycerin (*p*H 7.1). Stained preparations were observed with a Reichert Zetopan provided with ultraviolet illumination (14). FA staining reactions of bacterial cells were rated from 0 (no fluorescence) to 4+ (intense fluorescence with clearly defined outlines).

Specificity of conjugates. FA conjugates prepared by the two fractionation methods were tested against smears of various organisms from stock cultures, foodstuffs, infected tissues, intestinal tracts of rabbits and poultry, and soils. Direct smears of the last three materials were also stained with the FA conjugates and the conjugated preimmune serum. A supplemental test to prove the specificity of the conjugates was done by employing the inhibition test (13) which involved treatment of *S. necrophorus* smears with the homologous unlabeled antiserum before FA staining. Cultures showing positive reaction were compared with the test organism by immunodiffusion techniques and by disc gel electrophoresis (16).

Distribution of S. necrophorus in animal viscera and ruminal contents. Portions of the liver, gall bladder, spleen, mesenteric lymph node, small intestine, and ruminal content of five steers with liver abscesses and five apparently healthy steers were collected at the time of slaughter. The specimens were brought to the laboratory and processed aseptically for cultural and FA tests. Cultures containing FA-reactive cells were further screened to confirm their identity. Direct smears of the tissues and ruminal contents were examined similarly by the FA technique.

Soil studies. A freshly sampled pasture soil was passed through a 2-mm sieve and dispensed into screw-capped test tubes. Each tube contained 5 g of fresh soil. The soil contained 4.0% oxidizable organic matter and had a *p*H value of 6.6. Soil sterilization was achieved by autoclaving the tubes at 126 C for 20 min.

A two-part experiment was devised to evaluate the effect of incubation time and temperature on the antigenic reactivity of the test organism. The first part consisted of two sets of tubes incubated at 37 and 4 C. One set was kept anaerobic under a CO_2 plus N_2 atmosphere; to the other set about 1-cm layer of water was maintained above the soil surface. At monthly intervals, for 10 months, duplicate tubes were removed from each set and smears from diluted samples were subjected to direct FA examination.

In the second part of the experiment, autoclaved and unsterile soils were inoculated with the test organism and the moisture content was adjusted to 80% of the water-holding capacity (WHC). Both inoculated soils and the corresponding uninoculated controls were partitioned into four sets and incubated as follows: set 1, 37 C, N_2 plus CO_2 atmosphere; set 2, 37 C, air; set 3, 22 C, air; set 4, 4 C, air. Tubes were removed from each set at 0, 3, 5, 8, and 16 weeks and examined by FA procedures.

A short-term study was also undertaken to assess the relative survival of *S. necrophorus* in both sterile and unsterile soils. Samples were removed weekly and processed by cultural and FA methods for the presence of viable and fluorescing *S. necrophorus* cells. In a parallel experiment, heat-sterilized glass slides were buried in duplicate jars of inoculated sterilized and unsterile soils and incubated at 37 C. At weekly intervals, slides representing each treatment were removed and stained with the antibody conjugate and acridine orange (6).

RESULTS AND DISCUSSION

FA staining. *S. necrophorus* cells isolated from bovine liver abscesses reacted strongly with the labeled homologous antiserum. Figure 1 illustrates the typical intense fluorescence exhibited by actively growing *S. necrophorus* rods. Direct smears from 50 bovine liver abscesses obtained at different times invariably contained pleomorphic bacilli showing staining reactions of 3+ to 4+. A representative smear from an abscess (Fig. 2) demonstrates a cluster of fluorescing filaments among nonspecific fluorescing pus cells. The occurrence of nonspecific fluorescence by tissue cells and inert materials was of little consequence since the specific fluorescence of the test organism was quite intense and of a different color (bright apple green) than that of other materials (yellow, orange, red, or dull yellow-green). Absorbing the conjugates with dried liver powder (13) appeared to reduce the intensity of nonspecific reactions in tissue smears. Similarly, pretreating the smears with a gelatin preparation (3) before FA staining was effective in decreasing nonspecific fluorescence in soil smears and, to some extent, in tissue smears.

The agglutination titer of the antiserum was found to be 1:1,600. Five distinct precipitin bands were produced between the wells of the antiserum and the cytoplasmic fraction (18,000 × *g* supernatant fluid) by double agar-diffusion technique. Reactions between the antiserum and intact cells revealed three clear bands, whereas the crude cell wall fraction (18,000 × *g* pellet) elicited only one weak band. This observation suggests that *S. necrophorus* antigens prepared from disrupted cells might prove a better way of eliciting antibody response than using whole cell antigens.

Antibody conjugates derived from the two serum fractionation methods were both satisfactory and did not exhibit any obvious difference in staining the test organism. The rivanol fractionated antiserum, however, revealed fewer nonspecific reactions than antiserum precipitated directly with ammonium sulfate.

Specificity test. None of the heterologous bacterial species subjected to FA staining cross-reacted with the *S. necrophorus*-conjugated antiserum (Table 1). Fusiform bacilli cultures derived from a case of bovine foot-rot and a chronic foot infection showed strong staining reactions. These isolates were found to possess morphological,

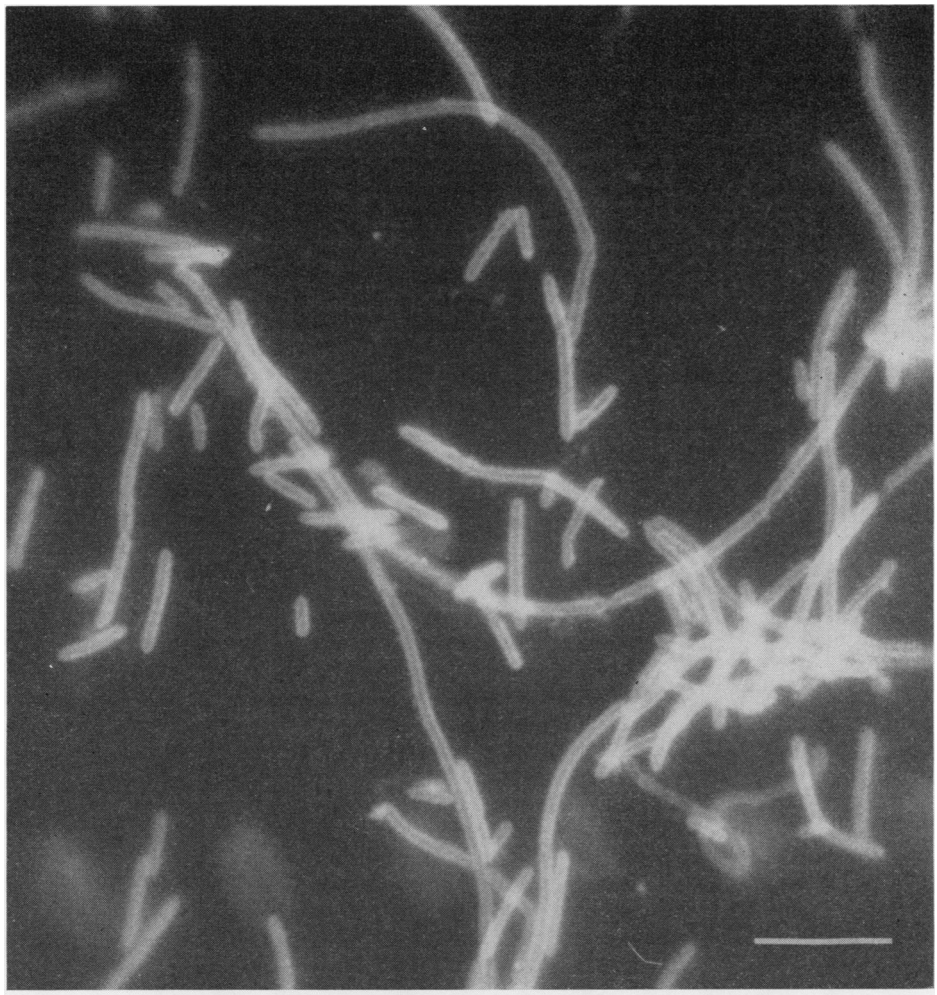

FIG. 1. *A 48-hr blood-agar culture of Sphaerophorus necrophorus stained with its homologous fluorescent antibody. White bar indicates 10 μm.*

biochemical, and pathogenic characteristics assigned to *S. necrophorus* species. Furthermore, results from a comparative gel diffusion test and disc gel polyacrylamide electrophoresis suggested the presence of certain antigens common to the foot isolates and the test organism. Thus, the *S. necrophorus*-conjugated antiserum appeared to be species-specific. Griffin (11) recently demonstrated similar FA reactions between *S. necrophorus* strains and suggested that there is more than one serotype within the species.

Drastic reduction of specific cell fluorescence in the inhibition test provided additional evidence for the specificity of the conjugated antiserum used in the study.

Distribution in the abdominal viscera and ruminal contents. Fluorescing *S. necrophorus* cells were detected in several organs of both healthy and diseased animals and in 4 of 10 ruminal contents examined (Table 2). The higher frequency of appearance of *S. necrophorus* cells in the liver and small intestines of diseased animals and their presence in healthy viscera suggest that the pathogen might gain entrance to the liver via the rumen and intestine. More cases, however, are needed to confirm this hypothesis. Smears of apparently healthy tissues found positive for *S. necrophorus* contained fluorescing rods which appeared shorter than the filamentous forms predominant in smears of liver abscesses. Inoculation of portions of these tissues to thioglycolate tubes, however, resulted in growth of long rods which subsequently showed strong FA staining.

Detection and survival in soil. Monthly FA

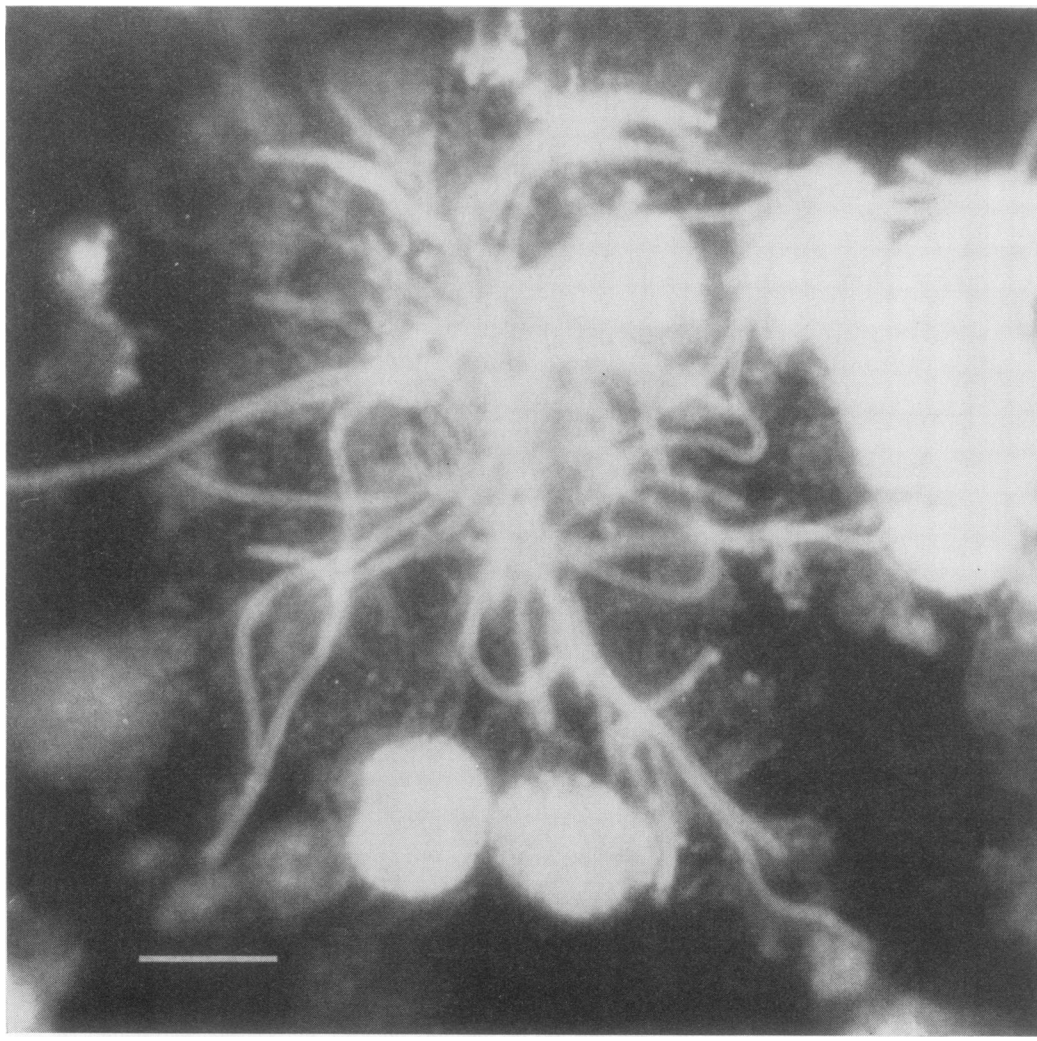

FIG. 2. *Direct smear of a bovine liver abscess stained with Sphaerophorus necrophorus fluorescent antibody, showing a cluster of S. necrophorus filaments against a background of nonspecifically stained pus cells. White bar indicates 10 μm.*

analysis of the anaerobically maintained inoculated soils indicated that *S. necrophorus* could retain its antigenic reactivity, as evidenced by fluorescing cells, for 8 and 10 months at 37 and 4 C, respectively. Unsterile soil produced a similar result (Table 3), although aerobic incubation at higher temperatures (37 and 22 C) effected a progressive reduction of the cell fluorescence after 8 weeks of incubation. The fluorescence of cells kept aerobic at 4 C and anaerobic at 37 C appeared to be maintained up to the last sampling date (16 weeks). When cultural and acridine orange staining methods were used as adjuncts to the FA technique, *S. necrophorus* was found to survive under both sterile and unsterile soil conditions for as long as 8 weeks (Table 4). Cells in unsterile soil, however, became progressively shorter starting at the 2nd week so that by the 8th week their length had decreased to about one-half to one-third that of cells inoculated to autoclaved soil. Coccoid forms of the test organism also appeared more numerous in the unsterile than the sterile soil. This morphological reaction might be a survival mechanism against an apparently competitive environment. The relatively prolonged survival in soil of *S. necrophorus* even under aerobic maintenance might be due to anaerobic pockets occurring in the lower portions

TABLE 1. *Some organisms tested against Sphaerophorus necrophorus conjugates*[a]

Organism	Staining reaction
Actinomyces bovis	0
Bacillus sp.	0
B. cereus	0
Bacteroides fragilis	0
B. melaninogenicus	0
Bacteroides sp.	0
Bordetella sp.	0
Brucella abortus	0
Clostridium chauvoei	0
C. septicum	0
Coliforms (mixed culture)	±
Corynebacterium sp.	0
C. pyogenes	0
Fusobacterium fusiforme	0
Fusobacterium sp.	0
Haemophilus suis	0
L2 (unknown species)[b]	0
L4 (unknown species)[b]	0
Listeria monocytogenes	0
Mycobacterium smegmatis (lysogenic strain)	0
M. smegmatis	0
M. tuberculosis	0
Pasteurella multocida	0
Proteus mirabilis	0
Staphylococcus aureus	0
Streptococcus sp.	±
Sphaerophorus necrophorus (bovine liver abscess isolates)	3+ to 4+
S. necrophorus (bovine foot-rot isolate)	3+
S. necrophorus (bovine chronic foot infection isolate)	3+
Sphaerophorus sp., isolate from normal rabbit small intestines (nonpathogenic)	0
Sphaerophorus sp., isolated from poultry ceca (nonpathogenic)	0
S. varius	0
Vibrio fetus	0
Eight gram-positive anaerobic isolates from three soils	0
Four gram-negative anaerobic isolates from three soils	0

[a] Conjugates used were from the rabbit antiserum.

[b] Organisms resembling L-forms isolated from bovine lymphosarcoma.

of the soil column and the relatively high soil moisture content (80% WHC). In a subirrigated swamp pasture, Marsh and Tunicliff (12) demonstrated the occurrence of foot-rot infection in sheep for 10 months but not for 20 months after the area has been heavily seeded with infectious

TABLE 2. *Distribution of Sphaerophorus necrophorus in the viscera and rumen content of cattle*

Specimen	No. of specimens showing positive fluorescence for *S. necrophorus*/no. of specimens examined	
	Healthy	Diseased[a]
Liver	1/5	3/5[b]
Gall bladder	1/5	1/5
Spleen	0/5	1/5
Mesenteric lymph node	1/5	1/5
Small intestine	1/5	3/5
Ruminal content	2/5	2/5

[a] Affected with liver abscess.

[b] Portion of liver free of abscess.

TABLE 3. *Effect of incubation temperature on the staining reaction of Sphaerophorus necrophorus in unsterile soil*[a]

Incubation time (weeks)	Staining reaction				
	Uninoculated controls	37 C anaerobic	37 C aerobic	22 C aerobic	4 C aerobic
0	0	4+	4+	4+	4+
3	0	4+	4+	4+	4+
5	0	4+	4+	4+	4+
8	0	4+	3+	4+	4+
16	0	4+	2+	3+	4+

[a] Soils were maintained at 80% water-holding capacity.

TABLE 4. *Relative survival and FA staining reaction of Sphaerophorus necrophorus in sterile and unsterile soils*[a]

Incubation time (weeks)	Viability and staining reaction		
	Uninoculated control	Inoculated sterile soil	Inoculated unsterile soil
0	0	V, 4+	V, 4+
1	0	V, 4+	V, 4+
2	0	V, 4+	V, 4+
3	0	V, 4+	V, 4+
4	0	V, 4+	V, 4+
5	0	V, 4+	V, 3 to 4+
6	0	V, 4+	V, 3+
7	0	V, 4+	V, 3+
8	0	V, 4+	V, 3+

[a] Soils incubated in air were maintained at 80% WHC.

[b] Viability (V) of the test organism was verified by subculturing in broth and by positive vital staining with acridine orange.

materials. Thus, the persistence of this pathogen in soil, although transitory, should not be overlooked particularly where recurrent foot-rot infections occur. Recent work (*unpublished data*) in this laboratory has shown the effectiveness of FA techniques in detecting *S. necrophorus* on the feet of both healthy and foot-rot-infected animals in various feedlots.

ACKNOWLEDGMENTS

We thank D. B. Way for technical assistance and B. Stewart for photomicrography. We also thank K. Malkin and S. M. Finegold, Veterans Administration Hospital, U.C.L.A., for providing some of the cultures used in this investigation.

LITERATURE CITED

1. Batty, I. 1967. Fluorescent-labelled antibodies as tools in the detection, identification and differentiation of clostridial species, p. 85–99. *In* V. Fredette (ed.), The anaerobic bacteria. The Institute of Microbiology and Hygiene, Univ. of Montreal.
2. Beveridge, W. I. B. 1934. A study of twelve strains of *Bacillus necrophorus* with observations of the oxygen intolerance of the organism. J. Pathol. Bacteriol. **38**:467–491.
3. Bohlool, B. B., and E. L. Schmidt. 1968. Nonspecific staining: its control in immunofluorescence examination of soil. Science **162**:1012–1014.
4. Breed, R. S., E. G. D. Murray, and N. R. Smith. 1957. Bergey's manual of determinative bacteriology, 7th ed. The Williams and Wilkins Co., Baltimore.
5. Calkins, H. E., and L. H. Schrivner. 1967. Isolation of *Sphaerophorus necrophorus* from bovine liver abscesses. Appl. Microbiol. **15**:1492–1493.
6. Casida, L. E. 1962. On the isolation and growth of individual microbial cells from soil. Can. J. Microbiol. **8**:115–119.
7. Dack, G. M., I. R. Dragstedt, and T. E. Heinz. 1937. Further studies on *Bacterium necrophorum* isolated from cases of ulcerative colitis. J. Infec. Dis. **60**:335–355.
8. Fievez, L. 1963. Etude comparee des souches de *Sphaerophorus necrophorus* isolees chez l'homme et chez l'animal. Presses Academiques Europeennes, Brussells.
9. Finegold, S. M., and L. G. Miller. 1967. Susceptibility to antibiotics as an aid in classification of gram-negative anaerobic bacilli. *In* V. Fredette (ed.), The anaerobic bacteria. The Institute of Microbiology and Hygiene, Univ. of Montreal.
10. Greenough, P. R. 1962. Observations on some of the diseases of the bovine foot. Part I. Vet. Rec. **74**:1–9.
11. Griffin, M. H. 1970. Fluorescent antibody techniques in the identification of the gram-negative nonsporeforming anaerobes. Health Lab. Sci. **7**:78–83.
12. Marsh, H., and E. A. Tunicliff. 1934. Experimental studies of foot-rot in sheep. Mont. Agr. Exp. Sta. Bull. **285**:3–16.
13. Nairn, R. C. 1964. Immunological tracing: general considerations, p. 103–137. *In* R. C. Nairn (ed.), Fluorescein protein tracing, 2nd ed. Williams and Wilkins, Baltimore.
14. Neil, D. H., M. M. Garcia, and K. A. McKay. 1970. A stable L-form of *Haemophilus pleuropneumoniae*. Can. J. Comp. Med. **34**:50–58.
15. Newsom, I. E. 1938. A bacteriologic study of liver abscesses in cattle. J. Infec. Dis. **63**:232–233.
16. Theodore, T. S., J. R. King, and R. M. Cole. 1969. Identification of L forms by polyacrylamide-gel electrophoresis. J. Bacteriol. **97**:495–499.