Comparative Tests for Detection of Plague Antigen and Antibody in Experimentally Infected Wild Rodents

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The enzyme-linked immunosorbent assay (ELISA) was compared with other standard tests for detection of plague (*Yersinia pestis*) antibody and antigen in multimammate mice (*Matomys coucha* and *M. natalensis*) which were experimentally infected and then killed at daily intervals postinoculation. For detection of antibody in sera from *M. natalensis*, the immunoglobulin G (IgG) ELISA was equivalent in sensitivity to passive hemagglutination and more sensitive than the IgM ELISA and complement fixation. Antibody was first detected on postinfection day 6 by all four tests, but IgM ELISA titers had declined to undetectable levels after 8 weeks. For detection of fraction 1 *Y. pestis* antigen in rodent organs, the ELISA was less sensitive than fluorescent antibody but more sensitive than complement fixation or immunodiffusion. Plague fraction 1 antigen was detected in 16 of 34 bacteremic sera from *M. coucha* and *M. natalensis*. The threshold sensitivity of the ELISA was approximately 10^5 *Y. pestis* per ml.

The enzyme-linked immunosorbent assay (ELISA) and radioimmunoassay (RIA) have been described for detection of plague (*Yersinia pestis*) antibodies in humans and animals (3, 9) and are being increasingly applied in routine serological diagnosis (9, 19) and surveillance (15, 18). Recent reports indicate that the ELISA and RIA may also be of importance for detection of plague fraction 1 (F1) antigen in sera of acutely ill patients (20) and in organs or rodents which succumb (16). In this communication, we extend the observations on the ELISA for detection of plague antibody and antigen in experimentally infected rodents by comparing its sensitivity with those of other standard tests.

MATERIALS AND METHODS

Plague strains and antigen. Virulent plague strain SAIMR 33243 isolated from a human in Ovamboland, South-West Africa (Namibia), was used in challenge experiments. Reference avirulent strain A1122 was used to prepare F1 antigen as described by Baker et al. (2) for isolation of soluble *Y. pestis* antigen. This procedure is essentially a salting-out technique using $(NH_4)_2SO_4$. The final precipitate was adjusted to a concentration of 4 mg of F1 antigen per ml and stored in 0.5-ml amounts at −70°C.

Antisera. Antibody to F1 antigen was produced in New Zealand White rabbits. Animals were initially immunized by subcutaneous inoculation with 1 mg of F1 antigen in complete Freund adjuvant at four sites and thereafter boosted with 0.5 mg of antigen alone at weekly intervals for 3 weeks. The rabbits were then sacrificed, and the sera were stored at −20°C.

Immune ascitic fluid against F1 antigen was prepared in mice as described by Sartorelli et al. (12). The mice were inoculated intraperitoneally with 0.5 mg of F1 antigen in complete Freund adjuvant at weekly intervals for 4 weeks. One week after the last inoculation, the mice were inoculated intraperitoneally with sarcoma 180 cells taken from a freshly killed mouse. When ascites was pronounced, the mice were killed and the fluid was harvested. The cells were

pelleted by centrifugation, and the supernatant mouse ascitic fluid was lyophilized in 0.5-ml volumes and stored at −20°C.

Animals. Multimammate mice, formerly *Praomys* (*Mastomys*) *natalensis*, comprise two sibling species in southern Africa with different karyotypes (6). The 2n = 32 chromosome species has been provisionally named *Mastomys natalensis*, and the 2n = 36 species has been named *M. coucha* (7). The value of multimammate mice as laboratory rodents has long been recognized (5), and colonies of both sibling species are maintained at this laboratory. The *Y. pestis* 50% lethal dose for each colony was previously found to be 1.4 × 10^3 organisms for *M. natalensis* and 7 organisms for *M. coucha* (14).

PHA and PHI tests. Passive hemagglutination (PHA) and passive inhibition (PHI) tests to detect plague antibody were performed by standard procedures recommended by the World Health Organization (1). Sheep erythrocytes were fixed in glutaraldehyde, sensitized with F1 antigen at a concentration of 400 μg/ml, and used in PHA and PHI tests at a final concentration of 0.6%.

Sera were screened for PHA antibody by preparing doubling dilutions in 25-μl volumes from 1:4 to 1:32 in 96-well U-bottom plates (Sterilin Ltd., Teddington, United Kingdom) and adding an equal volume of sensitized erythrocytes. The plates were incubated at room temperature for 3 h before being read. Samples showing hemagglutination were extended to endpoint in conjunction with a supplementary PHI control test. The PHI test was performed in the same way as the PHA test with the exception that F1 antigen was incorporated into the serum diluent at a concentration of 50 μg/ml. The serum-antigen mixture was incubated for 1 h at room temperature before addition of sensitized erythrocytes. The test was considered positive if the PHA endpoint titer was depressed eightfold, i.e., by 3 or more dilutions, in the PHI test. Sera with less than an eightfold difference in titer between PHA and PHI tests were considered nonspecific.

CF tests. Complement fixation (CF) tests were used to detect both antigen and antibody by procedures described by Bahmanyar and Cavanaugh (1). F1 antigen or mouse ascitic fluid was used at optimal dilution, and three lytic doses of

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complement were used. Sera were inactivated by incubation at 59°C for 30 min, and complement was fixed by overnight incubation at 4°C. Endpoints were recorded as the highest dilution of serum or antigen producing complete CF. Anticomplementary activity of samples was removed by incubation for 30 min at 37°C at a ratio of 1:1 with undiluted complement.

FA tests. Fluorescent antibody (FA) tests were performed to detect *Y. pestis* antigen in the organs of rodents which died or were sacrificed (11). Impression smears of tissues were made on microscope slides, fixed by immersion in 70% ethanol for 5 min, and air-dried. The smears were reacted with anti-F1 rabbit antisera for 30 min at 37°C, washed, and then reacted in the same manner with anti-rabbit fluorescein conjugate (Cooper Biomedical, Inc., West Chester, Pa.). After a final drying, the slides were examined with a UV light microscope.

ID tests. Immunodiffusion (ID) tests were used to detect F1 antigen in organ extracts, as described by Clarke (4), on microscope slides coated with 3 ml of 1% agarose dissolved in borate buffer, pH 9. Patterns of six peripheral wells and one central well were cut in the agar with a cutter and template kit (Miles Laboratories, Inc., Elkhart, Ind.). The central well was filled with anti-F1 antigen mouse ascitic fluid, and samples were screened undiluted in peripheral wells. Test samples were incubated at room temperature in humid containers and read at 24 and 48 h. Endpoints of positive specimens were determined in supplementary tests with doubling dilutions of sample.

ELISA. The ELISA procedure for detection of *Y. pestis* F1 antigen in organ extracts and sera was essentially similar to that of Williams et al. (20), and in preliminary tests it was found to be capable of detecting 8 to 16 ng of F1 antigen per ml. Rabbit anti-F1 hyperimmune serum at optimal dilution in carbonate-bicarbonate buffer (pH 9.6) was bound to 96-well ELISA plates by overnight incubation at 4°C. Plates were washed three times in phosphate-buffered saline, pH 7.1, containing 0.05% Tween 20 (PBST), and 50 µl of undiluted sample was added to appropriate wells. After incubation at 37°C for 30 min, the plates were washed three times in PBST and mouse ascitic fluid at optimal dilution in PBST was added to each well as an indicator antibody. The remainder of the test, with anti-mouse horseradish peroxidase conjugated immunoglobulin G (IgG), IgA, and IgM (Cooper Biomedical) and *o*-phenylenediamine as an indicator reagent, was performed as described previously (15). Results were determined by reading A_{492} on a Titertek Multiskan spectrophotometer (Flow Laboratories, Inc., McLean, Va.). Samples reacting positively in the screening test were extended to endpoint in supplementary ELISAs and subjected to ELISA inhibition (EI) tests to confirm the specificity of the reactions. In the EI test, samples were diluted in PBST containing rabbit anti-F1 antigen hyperimmune serum at a dilution of 1:100. Specific positive reactions showed a marked reduction in absorbance values in EI tests, whereas nonspecific samples showed almost identical values in the two tests. Endpoint titers of positive samples were defined as the highest dilution of antigen with at least a 50% higher absorbance value than the EI control in the ELISA. The ELISA and the EI test for detection of plague antibody were performed as described previously. Briefly, 50 µl of F1 antigen at a concentration of 10 µg/ml in carbonate-bicarbonate buffer was bound to each well of ELISA plates by overnight incubation at 4°C. Sample sera at 1:8 dilution in PBST were reacted with the plates for 30 min at 37°C. The remainder of the test was completed with the reagents and

procedures outlined above. Sera which reacted positively in the ELISA were extended to endpoint by testing doubling dilutions from 1:8 upward by ELISA and a concurrent EI test. In the EI test, samples were diluted in PBST containing 50 µg of F1 antigen per ml. The principle of the EI test and calculation of endpoint antibody titers were as described above for the antigen ELISA.

Extraction of antigen from organs. Soluble *Y. pestis* antigen was extracted from organs by the method of Larsen et al. (10). Liver and spleen tissues were homogenized to approximately 20% in sterile phosphate-buffered saline and treated with ether overnight, and the aqueous phase was clarified by centrifugation at 5,000 × *g* for 30 min.

Experimental infection of rodents. Adult *M. coucha* (*n* = 30) were inoculated subcutaneously with 150 virulent *Y. pestis*, and 5 to 10 animals were taken randomly and sacrificed daily. For measurement of bacteremia, whole heparinized blood was withdrawn by heart puncture and immediately diluted 10-fold to 10^{-7} in sterile saline. A total of 25 µl of each dilution was plated onto blood agar plates, and the plates were incubated for 48 h at 37°C for growth of *Y. pestis*. Impression smears of lung, liver, spleen, and lymph node tissues were made for FA tests, and spleen and liver tissues from animals which died were taken for extraction of *Y. pestis* soluble antigen. Sera were tested for presence of antibodies to *Y. pestis* by ELISA.

A total of 133 adult *M. natalensis* were inoculated subcutaneously with 10^3 virulent *Y. pestis*. Five animals were taken randomly and killed daily for the first 10 days and weekly thereafter for 8 weeks and tested for bacteremia and by FA tests as described above for *M. coucha*. Livers and spleens were removed from all animals killed and from three which died and stored at −70°C until treated for extraction of soluble *Y. pestis* antigen. Tissue extracts and sera were tested for presence of F1 antigen by the ELISA and CF and ID tests. Sera were also tested for plague antibody by the ELISA (IgG and IgM) and CF and PHA tests.

RESULTS

M. coucha. Five animals were killed at 24, 48, 62, 72, and 96 h, and 3 were killed at 120 h after inoculation. Two animals died on day 5 after inoculation. *Y. pestis* bacteremia was first detected in the blood of two animals killed at 62 h after inoculation and thereafter was detected in all animals tested, to titers as high as 10^8 bacteria per ml. Antibodies were not detected in sera, but F1 antigen was detected at titers ranging from 1:16 to 1:8,192 in 10 of the 15 bacteremic sera. The relationship between titers of bacteremia and ELISA F1 antigen for *M. coucha* and *M. natalensis* are presented in Fig. 1.

M. natalensis. A total of 46 *M. natalensis* died of plague between days 2 and 10. These animals were not used in the study, with the exception of three from which organs were removed and tested for the presence of F1 antigen. The results of antibody tests on sera from the animals which were bled daily and weekly are presented in Table 1. Antibodies were first detected in serum on day 6 by all four of the tests. Titers were highest by IgG ELISA and PHA and markedly lower by IgM ELISA and CF. A total of 33 sera were positive by PHA, 32 were positive by IgG ELISA, 23 were positive by CF, and 21 were positive by IgM ELISA. Of the 32 animals killed from weeks 2 to 8 after inoculation, 9 failed to seroconvert by any of the antibody tests.

Bacteremia was detected in the sera of 19 of the 55 animals which were killed on days 0 to 10 after inoculation. Of these
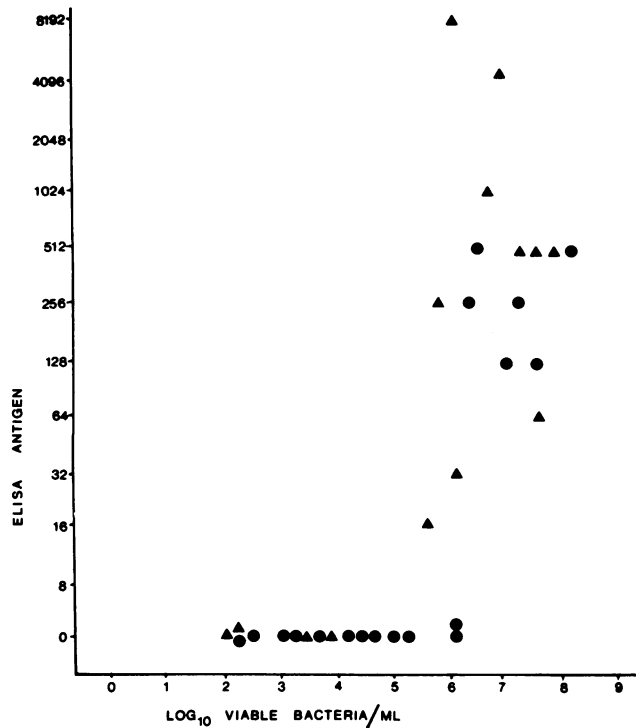
FIG. 1. Titers of plague F1 antigen detected by ELISA compared with *Y. pestis* titers in sera of bacteremic *M. coucha* (▲) and *M. natalensis* (●).

TABLE 2. Comparison of ELISA, CF, ID, and FA for detection of plague (*Y. pestis*) F1 antigen in organs of experimentally infected rodents

Rodent no.[a]	Reciprocal antigen titer by[b]:			
	ELISA	CF	ID	FA[c]
1	512	64	4	++
2	2,048	256	16	++
3	2,048	16	−	−
4	1,024	64	16	++
5	1,024	16	2	++
6	1,024	256	16	++
7	512	256	32	++
8	256	32	−	++
9	128	32	−	+
10	64	128	4	++
11	32	16	−	++
12	16	4	−	+
13	8	−	−	+
14	4	−	−	+
15	4	−	−	+
16–24	−	−	−	+

[a] Rodents number 1 to 7 died of plague, and numbers 8 to 24 were sacrificed at various times postinoculation. Number 1 was a white mouse, 2 and 3 were *M. coucha*, and all others were *M. natalensis*.

[b] Of the 24 animals tested, 15 were positive by ELISA, 12 were positive by CF, 7 were positive by ID, and 23 were positive by FA. The symbol − indicates a negative result.

[c] Results were based on the number of fluorescing bacteria per microscope field. Symbols: −, 0; +, 1 to 29; ++, 30 or more.

19, plague F1 antigen was detected by ELISA in the sera of 6 animals (Fig. 1).

Antigen detection in rodent organs. Table 2 summarizes the results of ID and CF tests and ELISA of tissue extracts and FA tests of organ impression smears from *M. coucha* and *M. natalensis* animals which died or were killed during the experiments. Antigen titers were highest in animals which died, and in such animals there was qualitative agreement

among the tests. Overall, antigen was detected by FA in 24 animals, by ELISA in 15, by CF in 12, and by ID in 7.

DISCUSSION

We previously reported that the PHA test and antibody ELISA were of similar sensitivity when used for serological plague surveillance in carnivores (15). In contrast, other workers reported that ELISA and RIA techniques were apparently more sensitive than PHA for serological diagnosis of plague in humans (9, 19). These discrepancies in the literature led us to suspect that there may be variation in the timing of the early antibody response when measured by different tests. The results of the present study, using *M. natalensis* as a laboratory rodent model, showed that onset of antibody response was detected simultaneously (day 6 postinoculation) by PHA, CF, IgG ELISA, and IgM ELISA. In itself, this is not surprising, since all of the tests use and therefore detect antibody to the highly purified F1 plague antigen. There were, however, differences in sensitivity among the tests. Antibody titers by PHA and IgG ELISA were comparable and considerably higher than those by CF or IgM ELISA. In addition, overall numbers of positive sera were greater by PHA and IgG ELISA than by CF or IgM ELISA. Suzuki et al. (17), using the PHA test, demonstrated in convalescent-phase sera of experimentally infected rats and rabbits that early sera were sensitive to 2-mercaptoethanol, which selectively degrades IgM, whereas later sera were insensitive. These workers showed that IgM antibody titers declined rapidly in sera of rats and rabbits, thus enabling determination of recent infection in field sera. In the present study, IgM ELISA titers had declined to negligible levels by 8 weeks postinfection, whereas IgG antibody was still present. This confirms that detection of IgM antibodies in convalescent-phase sera by ELISA is indicative of recent infection. However, because of the low concentrations of IgM antibody relative to IgG, it is conceivable that some

TABLE 1. Antibody response to subcutaneous infection with *Y. pestis* in *M. natalensis* determined by IgG and IgM ELISAs, PHA, and CF

Day postinoculation (no. of animals tested)	No. of animals seropositive (geometric mean titer)[a] by:			
	PHA	CF	IgG ELISA	IgM ELISA
0–5 (30)	0	0	0	0
6 (5)	3 (2.21)	1 (0.30)	2 (2.41)	3 (1.71)
7 (5)	2 (2.86)	1 (0.60)	2 (3.01)	1 (1.20)
8 (5)	2 (3.15)	2 (1.50)	2 (3.01)	1 (1.35)
9 (5)	2 (2.86)	2 (1.35)	2 (2.86)	1 (1.35)
10 (5)	1 (2.71)	1 (1.20)	1 (2.71)	2 (1.81)
14 (5)	4 (2.05)	3 (0.97)	4 (2.48)	3 (1.28)
21 (5)	2 (3.15)	2 (1.65)	2 (3.15)	2 (2.30)
28 (5)	4 (2.40)	3 (1.05)	4 (2.30)	2 (1.13)
35 (5)	5 (2.34)	3 (0.90)	5 (2.50)	2 (0.72)
42 (5)	3 (2.50)	3 (1.28)	3 (2.10)	3 (1.13)
49 (4)	3 (2.10)	1 (0.60)	3 (2.30)	1 (0.50)
56 (3)	2 (1.95)	1 (0.75)	2 (2.26)	0

[a] The overall geometric means (± standard error of the mean) titers were as follows: PHA, 2.44 ± 0.10; CF, 0.98 ± 0.13; IgG ELISA, 2.53 ± 0.12; IgM ELISA, 1.15 ± 0.17.

early convalescent-phase sera may be IgG positive but IgM negative.

Using monoclonal antibody as a capture antibody and purified rabbit immunoglobulin as an indicator antibody, Williams et al. (20) described an ELISA capable of detecting 8 ng of F1 antigen per ml. The system described here was found to be of an equivalent sensitivity despite the use of crude, unpurified hyperimmune mouse ascitic fluid and rabbit antisera. However, in contrast to Williams et al. (20), we found it necessary to use a criterion of a 50% difference between values in the ELISA and EI test to ensure specificity of positive samples. When we compared ELISA antigen titers relative to bacteremic titers in *M. coucha* and *M. natalensis* sera, F1 antigen was regularly detected in sera with bacteremic titers of $>10^5$ *Y. pestis* per ml (approximately equivalent to 5×10^3 bacteria per 50-μl sample as used in the test). The sensitivity was similar when testing 24-h broth cultures of *Y. pestis* SAIMR 33243 and A1122 (data not shown) and appeared to be only slightly less sensitive than the RIA technique described by Soergel et al. (16).

In comparative tests for detection of F1 antigen in tissue extracts, ELISA was less sensitive than the FA test but more sensitive than CF or ID (Table 2). Under ideal conditions, all four techniques could be used for detection of antigen in rodent carcasses. In many cases, however, rodent corpses are received for plague investigation in an advanced stage of decomposition, rendering isolation of *Y. pestis* difficult by culture or animal inoculation. Although it is the most sensitive technique of F1 antigen detection, the FA test is not quantitative and becomes progressively more difficult to interpret with decomposition of carcasses, owing to lysis of bacteria (8). In such cases, the ELISA has advantages over the other quantitative tests (ID and CF) in that it is more sensitive and specific and can be completed in only 2 to 3 h.

In South Africa, direct detection of epizootic plague is difficult owing to rapid removal of rodent carcasses by scavenging carnivores. Moreover, routine collection of rodent organs in the field for attempted isolation is time-consuming and has proved of little practical value owing to apparently low infection rates in wild rodent populations (13). Currently, therefore, plague surveillance is reliant on antibody surveys in dogs and wild rodents (15). We have found (unpublished data) that on infection with *Y. pestis*, *M. natalensis* either (i) survives with or without seroconversion after mild bacteremia or without developing bacteremia or (ii) dies after severe bacteremia of 1 to 3 days duration. The results of the present experiments suggest that testing of rodent sera for *Y. pestis* antigen by ELISA would be likely to detect only those individuals with severe ($>10^5$ bacteria per ml), ultimately fatal bacteremia. Screening of rodent sera from the field by ELISA for plague antigen, as well as antibody, may therefore offer a convenient and simple method for early detection of rodent epizootics.

LITERATURE CITED

1. Bahmanyar, M., and D. C. Cavanaugh. 1976. Plague manual. World Health Organization, Geneva.
2. Baker, E. E., H. Sommer, L. E. Foster, E. Meyer, and K. F. Meyer. 1952. Studies on immunity against plague. I. The isolation and characterization of the soluble antigen of *Pasteurella pestis*. J. Immunol. 68:131–145.
3. Cavanaugh, D. C., M. K. Fortier, D. M. Robinson, J. E. Williams, and J. H. Rust, Jr. 1979. Application of the ELISA technique to problems in the serologic diagnosis of plague. Bull. Pan Am. Health Organ. 13:399–402.
4. Clarke, D. H. 1964. Further studies on antigenic relationships among the viruses of the group B tick-borne complex. Bull. W.H.O. 31:45–56.
5. Davis, D. H. S., and A. G. Oettle. 1958. The multimammate mouse *Rattus* (*Mastomys*) *natalensis* Smith: a laboratory-adapted African wild rodent. Proc. Zool. Soc. London 131:293–299.
6. Green, C. A., D. H. Gordon, and N. F. Lyons. 1978. Biological species in *Praomys* (*Mastomys*) *natalensis* (Smith), a rodent carrier of Lassa virus and bubonic plague in Africa. Am. J. Trop. Med. Hyg. 27:627–629.
7. Green, C. A., H. J. Keogh, D. H. Gordon, M. Pinto, and E. K. Hartwig. 1980. The distribution, identification and naming of the *Mastomys natalensis* species complex in southern Africa. J. Zool. (London) 192:17–23.
8. Hudson, B. W., S. F. Quan, and L. Kartman. 1962. Efficacy of fluorescent antibody methods for detection of *Pasteurella pestis* in carcasses of albino laboratory mice stored for various periods. J. Hyg. 60:443–450.
9. Hudson, B. W., K. Wolff, and T. Butler. 1980. The use of solidphase radioimmunoassay techniques for serodiagnosis of human plague infection. Bull. Pan Am. Health Organ. 14:244–250.
10. Larsen, C. L., C. B. Philip, W. C. Wicht, and L. E. Hughs. 1951. Precipitin reactions with soluble extracts from suspensions of *Pasteurella pestis* or from tissues of animals dead of plague. J. Immunol. 67:289–298.
11. Moody, M. D., and C. C. Winter. 1959. Rapid identification of *Pasteurella pestis* with fluorescent antibody. III. Staining *Pasteurella pestis* in tissue impression smears. J. Infect. Dis. 104:288–294.
12. Sartorelli, A. C., D. S. Fischer, and W. G. Downs. 1966. Use of sarcoma 180/TG to prepare hyperimmune ascitic fluid in the mouse. J. Immunol. 96:676–682.
13. Shepherd, A. J., and P. A. Leman. 1985. Bacterial surveillance of South African rodents. South Afr. J. Sci. 81:302–308.
14. Shepherd, A. J., P. A. Leman, and D. E. Hummitzsch. 1986. Experimental plague infection in South African wild rodents. J. Hyg. 96:171–183.
15. Shepherd, A. J., P. A. Leman, D. E. Hummitzsch, and R. Swanepoel. 1984. A comparison of serological techniques for plague surveillance. Trans. R. Soc. Trop. Med. Hyg. 78:771–773.
16. Soergel, M. E., F. L. Schaffer, and H. F. Blank. 1982. Solid-phase radioimmunoassay for detection of plague antigen in animal tissue. J. Clin. Microbiol. 16:953–956.
17. Suzuki, S., Y. Chikasato, and S. Hotta. 1974. Studies on antiplague haemagglutinating antibodies. Treatment of test sera with acetone and 2-mercaptoethanol. Bull. W.H.O. 51:237–243.
18. Willeberg, P. W., R. Ruppanner, D. E. Behymer, H. H. Higa, C. E. Franti, R. A. Thompson, and B. Bohannan. 1979. Epidemiologic survey of sylvatic plague by serotesting coyote sentinels with enzyme immunoassay. Am. J. Epidemiol. 110:328–334.
19. Williams, J. E., L. Arntzen, D. M. Robinson, D. C. Cavanaugh, and M. Isaacson. 1982. Comparison of passive haemagglutination and enzyme-linked immunosorbent assay for serodiagnosis of plague. Bull. W.H.O. 60:777–781.
20. Williams, J. E., M. K. Gentry, C. A. Braden, F. Leister, and R. H. Yolken. 1984. Use of an enzyme-linked immunosorbent assay to measure antigenaemia during acute plague. Bull. W.H.O. 62:463–466.