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Serological Diagnosis of Human Melioidosis with Indirect Hemagglutination and Complement Fixation Tests

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An indirect hemagglutination (IHA) test and a complement fixation (CF) test were evaluated from test results on sera from 212 human melioidosis patients of which 119 were culturally proved cases. Significant antibody titers (IHA titers of 1:40 or greater and CF titers of 1:4 or greater) were demonstrated with either test in all except five patients. IHA and CF titers ranged as high as 1:20,480 and 1:1,024, respectively. Antibodies were usually demonstrated by both tests 1 week after onset of disease. Transient seronegative reactions during the course of disease were seen in sera of approximately 19% of the patients with either IHA and CF but rarely with both tests. High titers in either test were obtained by the third week of disease and reached maximum levels in 4 to 5 months. Titers usually were detectable for 9 or more months. Antibodies were detected by IHA and CF tests in 80 to 100% of the sera obtained at various time intervals from 9 months to 2 or more years after disease onset. Antibody persistence occurred in patients who had a short disease course, as well as in patients with prolonged, complicated infections. The IHA test had excellent specificity when evaluated with normal human sera and diverse antimicrobial sera from hyperimmunized rabbits and human patients. The CF antigen appeared to contain common antigens with some but not all types of *Pseudomonas aeruginosa*. The specificity of the CF antigen could be enhanced without appreciable effect on its sensitivity by use of a titer of 1:8 in lieu of 1:4 as a criterion for a significant reaction. Either test could be used advantageously for the laboratory diagnosis of melioidosis.

Melioidosis, an infectious disease caused by *Pseudomonas pseudomallei*, is endemic to Southeast Asia. It has been increasingly recognized in U.S. Armed Forces personnel who have served in South Vietnam (1, 3, 6, 17, 20). The disease signs are not pathognomonic. Manifestations may range from a mild subacute disease with localized lesions to a rapidly fatal septicemic form. Dormant or inapparent infections occur and may recrudesce in severe fulminant form months and even years after exposure (4, 14). The diagnosis of melioidosis can only be established by isolation and identification of the etiological agent or by the serological demonstration of specific antibodies. The importance of early recognition and institution of appropriate therapy for the successful management of this disease has been emphasized (1, 3).

Since 1964, the indirect hemagglutination (IHA) test of Ileri (8) and a complement fixation (CF), similar to that of Nigg and Johnston (13), have been used effectively by this laboratory for the diagnosis of melioidosis. The sensitivity and specificity of the IHA and CF tests for the serological diagnosis of melioidosis are reported.

MATERIALS AND METHODS

Source of sera. A total of 750 sera were derived from 212 human melioidosis patients. Sera were submitted with few exceptions from various U.S. Armed Forces medical installations in Southeast Asia and the United States from November 1964 to July 1969. Samples from two patients were submitted prior to 1964 and were retested. The 210 cases diagnosed after 1964 comprised 201 U.S. Armed Forces personnel,

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TABLE 1. *Distribution of 212 cases of melioidosis according to diagnostic criteria*

Diagnostic criteria	Clinical history	Laboratory data only	Total
Isolation of *P. pseudomallei*	113	6	119
Significant titers in IHA and CF tests	54[a]	32[b]	86
Significant titers in IHA test	3[c]	4[d]	7
Total	170	42	212

[a] Distribution of maximum titers. IHA test: 1:40 (3); 1:80 (5); 1:160 (6); 1:320 (8); 1:640 (9); 1:1280 and greater (23). CF test: 1:8 (3); 1:16 (11); 1:32 (5); 1:64 (8); 1:128 and greater (27).

[b] Distribution of maximum titers. IHA test: 1:40 (3); 1:160 (3); 1:320 (7); 1:640 (4); 1:1,280 and greater (15). CF test: 1:16 (4); 1:32 (5); 1:64 (5); 1:128 and greater (18).

[c] Distribution of maximum titers. IHA test: 1:160 (1); 1:1,280 (2). CF test: not done or anticomplementary.

[d] Distribution of maximum titers. FUO cases with demonstrable titer conversions. IHA test: 1:160 (1); 1:1,280 (3); CF tests not done or anticomplementary.

of whom 198 had served in South Vietnam and 3 in Thailand, 4 British Commonwealth soldiers in Malaysia, and 5 Vietnamese civilian or military personnel.

The laboratory diagnoses of 42 patients were initially established at the 9th U.S. Army Medical Laboratory in Saigon with the IHA test. Duplicate samples from these patients were retested with IHA and CF procedures at Walter Reed Army Institute of Research (WRAIR). The IHA test findings in the two laboratories were essentially the same.

The distribution of 212 cases according to diagnostic criteria is summarized (Table 1). Melioidosis infections of 119 patients were proved bacteriologically by cultural recovery and identification of *P. pseudomallei*, and with two exceptions, also by serological tests. The remaining 93 patients were admitted as melioidosis cases primarily on the basis of serological findings. The clinical histories of 57 of the 93 patients were known and consistent with a diagnosis of melioidosis. Thirty-two patients whose clinical histories were unknown had high-titer serum antibodies by both IHA and CF tests. The criteria for significant reactions in IHA and CF tests were based on observed reactions in proved cases of melioidosis, reactions of comparable groups of normal persons with no history of exposure to melioidosis, and reactions of patients in U.S. hospitals who were either convalescing from or were in secondary stages of various diseases. The patients were predominantly soldiers with no history of residence in Southeast Asia. The specificity of the IHA and CF test was also evaluated with diverse antimicrobial hyperimmune rabbit sera obtained from various laboratories.

The distribution of 212 cases by types of disease was as follows: localized pulmonary infections, 105; pulmonary and other tissue infections, 14; localized wound or burn infections, 25; wound and other tissue infections, 5; septicemia, 10; prostatitis, 1; localized subcutaneous infections, 2; osteomyelitis, 1; appendicitis, 1; pericarditis, 1; lymphadenopathy, 1; hepatitis, 4; fevers of unknown origin, 12; and unknown, 30.

Source of strains. Antigens for both IHA and CF tests consisted of pooled preparations from three different strains of *P. pseudomallei*. Various combinations of strains were tested and used at different times for the two tests. There was no evidence that differences in strain combinations had a bearing on the sensitivity and specificity of the IHA and CF tests. The IHA testing of most of the sera in this study was done with *P. pseudomallei* pooled antigens from strains 8202, China 3, and 295. CF tests have been primarily conducted with pooled antigens from strains 8202, China 3, and 7820 in lieu of 295. The source of antigen strains is as follows. (i) *P. pseudomallei* strain 8202 was isolated September 1966 from sputum of a soldier with a localized pulmonary infection contracted in Vietnam. (ii) *P. pseudomallei* strain China 3 was from the stock culture collection of WRAIR. It was received in 1945 and is probably an isolate recovered from a fatal septicemia case in an American soldier in Burma. (iii) *P. pseudomallei* strain 295 has an unknown source. Old stock culture was from the Calcutta School of Tropical Medicine. (iv) *P. pseudomallei* strain 7820 was isolated in March 1966 from an infected gunshot wound on the finger of an American soldier evacuated from Vietnam.

IHA test. The IHA test was a modification of the procedure described by Ileri (8). The test was initially conducted in hemagglutination test tubes and later adapted for use with the microtiter technique. The sensitivity and specificity of the two techniques were found to be essentially the same when comparative tests were done on 50 sera, equally derived from known infected and normal human beings. Details on the preparation and standardization of antigen, preparation of sensitized sheep cells, and conduct of tube IHA test were reported previously (18). Generally, antigen for each of the three strains was prepared by the method of Rice et al. (15), and consisted of the supernatant fluid of a heat-killed 2-week-old protein-free broth culture. The antigen was preserved by addition of phenol (0.5% by volume) and was stored at 4 C. Antigens have maintained their activity over a 3-year observation period. Optimum dilutions of each antigen for sensitizing sheep red blood cells were predetermined by appropriate block titrations of reference antiserum and antigen and pooled in equal volumes. An anti-*P. pseudomallei* rabbit serum was first used as a reference antiserum and later replaced with a high-titer convalescent serum from a proved human case of melioiodosis. The optimum antigen dilution with either the rabbit or human antiserum was usually 1:100. Washed sheep red blood cells were sensitized by the addition of 10 volumes of pooled

antigen to 1 volume of a 10% suspension of cells. After 1 hr of incubation at 37 C, the sensitized cells were centrifuged, washed once with physiological salt solution, and resuspended to 1% concentration in saline diluent. Test sera previously inactivated (56 C) and adsorbed with nonsensitized sheep red blood cells were serially diluted twofold starting with a 1:10 or 1:20 dilution. Phosphate-buffered physiological salt solution, *p*H 7.2, containing 0.06% bovine serum albumin was used as diluent. To 0.4 ml of each serum dilution, 0.1 ml of 1% sensitized erythrocytes was added in a hemagglutination tube. Tubes were kept at room temperature, shaken at intermittent intervals, and examined for agglutination after 2 and 18 hr. The titers at the two reading times were essentially the same. Normal serum, antigen, cell diluent, and antiserum controls were used for each series of tests. The titer was the highest dilution of serum which distinctly agglutinated sensitized erythrocytes. In the microtiter IHA technique, inactivated and adsorbed sera were serially diluted twofold in microtiter plates with a 0.05-ml loop, starting with a 1:10 dilution. One per cent suspension of sensitized sheep erythrocytes was added to each serum dilution with a 0.025-ml dropper. Appropriate controls were used as in the tube test. Plates were sealed with transparent cellophane tape, shaken vigorously, and held at room temperature for 2 hr and then examined with the aid of a microtiter test reading mirror. Positive reactions were manifested by an agglutinated mat across the bottom of the well. The edges of the mat were frequently folded. In some cases agglutinated cells formed a tightly clumped mass in the center of the well giving the impression of a "button"; however, irregular edges were discernible upon close examination, and distinct clumps were seen when the plate was shaken. When reactions were negative, there was a distinct red "button" in the bottom of the well. In intermediate or partial reactions, the "button" was broader. The titer was the highest dilution in which a positive reaction was demonstrated.

CF test. Preparation of CF antigen was similar to that described by Nigg and Johnston (13). Antigens from three strains were prepared separately, and predetermined optimum dilutions were pooled in equal volumes to provide the test antigen. Each strain was grown on the surface of brain-heart infusion agar (*p*H 6.8) in ten 32-oz prescription bottles, each of which was seeded with 2.0 ml of a heavy suspension of organisms. The seed suspension was prepared from the 24- to 48-hr smooth confluent growth on a petri plate culture. After 48 hr of incubation at 35 to 37 C, growth in each bottle was suspended in 20 ml of sterile distilled water, subcultured for purity determinations, and stored at 4 C. If cultural tests were satisfactory, suspensions were pooled. The pooled suspension was standardized spectrophotometrically to a density of approximately 14×10^9 cells/ml by optical density (OD) measurements at 610 μg of appropriate dilutions and reference to a previously determined standard curve relating bacterial counts to OD. The standardized suspensions were inactivated by heating in a 65 C water bath for 1 hr, and then subcultured for sterility determinations and stored at 4 C. Subcultures were incubated for 4 days at 35 C to ascertain the absence of viable organisms in the heat-treated cell suspension. Cells were disrupted by subjecting 70-ml amounts of standardized cell suspension, in a 125-ml oscillator cup, to sonic vibration at a frequency of 20 kc/sec for 10 min in a 4 C water-cooled oscillator (Biosonek) at a maximum output of 600 w. The sonically treated suspensions were centrifuged in a 4 C refrigerated centrifuge at 18,000 $\times$ *g* for 30 min, and the supernatant fluid was collected. The sediment was resuspended in water to one-fourth of the original volume of the standardized suspension and centrifuged, and the supernatant fluid was combined with that of the first centrifugation. The combined supernatant fluids were successively filtered through 0.8- to 1.2-μm and 0.22- or 0.45-μm pore size sterile filters (Millipore Corp., Bedford, Mass.). The filtered supernatant fluids, which constituted the stock CF antigen, were distributed in sterile vaccine bottles and stored at 4 C. Stored antigens have remained stable over a 3-year observation period.

The procedures described by Kent and Fife (9) were followed for preparation and standardization of sheep erythrocytes and standardization of complement, hemolysin, and antigen. The reagent diluent was triethanolamine-buffered salt solution. One liter of stock solution (10$\times$ concentrated) contains 75 g of NaCl, 28 ml of $N(CH_2CH_2OH)_3$, 170 ml of 1 N HCl, 1 g of $MgCl_2 \cdot 6H_2O$, and 0.2 g of $CaCl_2 \cdot 2H_2O$. The optimum dilution of *P. pseudomallei* antigen for conducting the test varied from 1:100 to 1:300. Reference antisera were the same as those used for IHA tests.

Test sera were inactivated at 56 C for 30 min and serially diluted twofold from 1:4 through 1:64 or greater if necessary. To 0.3 ml of diluted serum, 0.3 ml of a solution containing five (50%) hemolytic units of complement and 0.3 ml of pooled antigen were added in a standard Wasserman tube. Mixtures were held at 2 to 4 C for 16 hr and then warmed to room temperature, and 0.6 ml of sensitized sheep erythrocytes was added. Test tubes were held at 37 C for 30 min and then examined. Results were recorded in terms of degree of fixation from negative to 4+. The titer was the highest dilution of serum with a 2+ (e.g., 50% hemolysis) or greater reaction. Appropriate controls were used for each series of tests.

RESULTS

Specificity tests. The specificity of the IHA test was initially evaluated with sera submitted to this laboratory from various military and other medical installations in the United States, primarily for serological tests for toxoplasmosis and leptospirosis. Seven of 200 sera were reactive, four at titer of 1:10 and three at titer of 1:20 (18). On the basis of these findings, titers of 1:40 were considered to be positive for evaluation of prevalence of antibody in indigenous populations of an endemic area of melioidosis (18). Results

TABLE 2. *IHA and CF tests on sera from persons with no history of exposure to melioidosis*

Group	No. reactive/no. tested		Distribution of reactions
	IHA	CF	
U.S. Government employees	5/145	NT[a]	1:20 (3); 1:40 (2)
U.S. Government employees	NT	1/100	1:2
Army nursing service personnel	0/27	0/26	
Soldiers	0/500	NT	

[a] Not tested.

of additional tests on different population groups with no history of infection with *P. pseudomallei* served to affirm the selection of this titer as a criterion for significant serological reaction (Table 2). Nonspecific reactions in normal persons in the United States, as well as in soldiers first entering Vietnam, did not occur at serum dilutions greater than 1:40. The percentages of positive IHA reactions did not exceed 3.4% at 1:20 and 1.7% at 1:40 serum dilutions.

Only one CF reaction with a titer of 1:2 was elicited in a total of 126 sera from two different groups of serum samples drawn from persons with no history of exposure to melioidosis (Table 2). This finding was consistent with that of Nigg (12), who found no CF antibodies in 138 sera obtained from persons in the United States.

Results of IHA and CF tests on diverse antimicrobial rabbit sera are shown (Table 3). IHA reactions with 16 different *P. pseudomallei* antisera varied from 1:2,560 to 1:40,960. Comparable high-titer reactions obtained with three *Actinobacillus mallei* antisera were expected in view of the close biological relationship of the two species (16). Otherwise, there were few low-titer (1:20 to 1:40) cross-reactions even in the large series of *P. aeruginosa* antisera, which were prepared from representative strains of various serotypes described by Verder and Evans (19) and Habs (7), as well as of different phage types.

CF test titers of 19 *P. pseudomallei* and 3 *A. mallei* antisera were 1:1,024. One anti-*A. mallei* serum had a titer of 1:512. Heterogenous reactions of CF antigen were difficult to evaluate with hyperimmune rabbit serum because of anticomplementary reactions. These untoward reactions obviated determinations of low-titer cross-reactions of 1:4 to 1:16 in approximately 40% of 166 antisera prepared from taxonomically unrelated strains. When satisfactory tests were obtained, CF titers varying from 1:4 to 1:64 were commonly seen in various antimicrobial as well as normal rabbit sera. There was ancillary evidence that common antigen-antibody factors figured in cross-reactions of CF antigen with different *P. aeruginosa* antiserum. A fourfold or greater rise in titer was demonstrated in pre- and postimmune sera of 7 of 12 rabbits immunized with different Verder and Evans (19) serotype strains. Satisfactory CF tests were not obtained on paired sera from six other immunized rabbits in this group. No clues were evident on the nature of other cross-reactions.

Tests conducted on sera from patients with various diseases other than melioidosis afforded additional evidence of occurrence of specific cross-reactions with some *P. aeruginosa* antibodies in the CF test (Table 4). Four of ten patients with *P. aeruginosa* infections had relatively high CF test titers ranging from 1:8 to 1:128 but were negative with the IHA test. A patient with *P. stutzeri* infection had a CF titer of 1:32 and an IHA titer of 1:40. Other relatively high CF titers (1:8 and greater) were seen in sera from a leptospirosis patient, who also had a IHA titer of 1:20, and in two influenza patients. Except as noted, the reactors with the CF test did not have antibodies with the IHA test at a serum dilution of 1:20 or greater. The overall percentage of CF reactions in this series of nonmelioidosis patients was 11% and was in marked contrast to the paucity of CF reactions in sera from normal individuals.

The prevalence of IHA test reactions in nonmelioidosis patients was 10% (Table 4). Although the percentage of reactions was higher than that obtained with sera from normal individuals, the titers were low-order reactions of 1:20 to 1:40.

IHA and CF tests on melioidosis patients. IHA tests were done on all except five sera, but all patients were tested. CF tests were completed on one or more sera from 203 patients. CF tests on 76 sera were either unsatisfactory or were not done. On the basis of test findings on sera of normal human beings and nonmelioidosis patients, minimum titers of 1:40 in the IHA test and 1:4 in the CF test were considered to be significant. IHA and CF titers ranged as high as 1:20,480 and 1:1,024, respectively.

Serological findings on bacteriologically verified cases were used to evaluate and compare the sensitivity of IHA and CF tests for laboratory diagnosis of melioidosis. Appropriate comparative data were available for 455 sera from 114 cases (Table 5). Significant antibody titers in one or both tests were found in one or more serum samples from all but 2 of 114 patients. The two

TABLE 3. *Reactions of hyperimmune rabbit sera for diverse microorganisms in the IHA and CF tests for melioidosis*

Type of antibody	No. of reactions/no. tested		Distribution of reactions
	IHA	CF	Test titer (no.)
Pseudomonas pseudomallei	16/16	19/19	IHA–1:2,580 (3); 1:10,240 (3); 1:20,480 (5); 1:40,960 (5) CF –1:1,024 (19)
Actinobacillus mallei	3/3	4/4	IHA–1:2,560 (2); 1:20,480 (1); CF –1:512 (1); 1:1,024 (3)
A. lignieresii	0/23	2/2	CF –1:8 (2)
Salmonella sp.	0/9	2/6	CF –1:8 (2)
S. typhosa	0/2	1/2	CF –1:8
Escherichia coli	0/2		
Shigella sp.	0/5		
Proteus sp.	0/3		
Pasteurella tularensis	0/1	1/1	CF –1:16
P. pestis	0/2		
P. pseudotuberculosis	0/1	1/1	CF –1:16
P. multocida	0/1	1/1	CF –1:16
Vibrio cholera	0/1	1/1	CF –1:16
Mima sp.	0/2	2/2	CF –1:16; 1:32
Leptospira sp.	0/2	2/2	CF –1:8; 1:32
Listeria monocytogenes	3/5		IHA–1:20 (3)
Brucella abortus	0/1	1/1	CF –1:8
B. bronchiseptica	0/3	1/1	CF –1:16
Pseudomonas aeruginosa[a]	2/60	12/22	IHA–1:20 (2) CF –1:4 (5); 1:8 (4); 1:16 (3)
P. aeruginosa (preimmune)[b]	2/17	4/12	IHA–1:20 CF –1:8; 1:16 (2); 1:32
P. aeruginosa (postimmune)[b]	0/18	14/14	CF –1:8 (2); 1:16 (8); 1:32 (2); 1:64 (2)
P. stutzeri	1/5	2/2	IHA–1:40 CF –1:8; 1:16
P. multivorans	1/3		IHA–1:20

[a] Includes Verder and Evans, Habs, and other (e.g., phage) types.
[b] Verder and Evans serotypes. Seven rabbits had a fourfold or greater increase in CF titer.

TABLE 4. *IHA and CF tests for melioidosis on sera from nonmelioidosis patients*

Disease	No. of reactions/no. tested		Distribution of reactions
	IHA	CF	Test titer (no.)
Tuberculosis	1/30	1/30	IHA–1:40; CF–1:4
Pseudomonas aeruginosa infection	1/12	4/10	IHA–1:20; CF–1:8 (1); 1:64 (1); 1:128-(2)
P. stutzeri infection	1/1	1/1	IHA–1:40; CF–1:32
Leptospirosis	2/12	2/8	IHA–1:20 (2); CF–1:4, 1:64
Brucellosis	0/1	1/1	CF –1:4
Typhoid	0/1	1/1	CF –1:4
Typhus	3/3	0/1	IHA–1:40 (3)
Influenza	1/4	2/3	IHA–1:40; CF–1:8, 1:16
Lymphogranuloma venereum	1/6	0/5	IHA–1:20
Miscellaneous respiratory tract infections	1/6	0/6	IHA–1:20
Other[a]	0/28	0/23	
Unspecified[b]	1/16	0/16	IHA–1:20
Totals	12/122	12/107	IHA–1:20 (6); 1:40 (6) CF –1:4 (4); 1:8 (2); 1:16 (1); 1:32 (1); 1:64 (2); 1:128 (2)

[a] Tularemia, 0/1; cholera, 0/1; salmonellosis, 0/1; syphilis, 0/2; infectious hepatitis, 0/1; primary atypical pneumonia, 0/1; malaria, 0/15; asthma, 0/3; rheumatic fever, 0/1 (HA test only); rheumatoid arthritis, 0/4 (HA test only).
[b] Cases from Medical Ward, Valley Forge General Hospital, Phoenixville, Pa.

TABLE 5. *Comparison of IHA and CF results on 455 sera from 114 bacteriologically verified cases of melioidosis*

Complement fixation text	Reactions			
	IHA test (455 sera)		IHA test (114 patients[a])	
	Negative	Positive	Negative	Positive
Negative	12	18	2[b]	1
Positive	41	384	3	108
Correlation	396/455	87.0%	110/114	96.5%

[a] Sera from five additional patients were positive on IHA test but were not tested with CF test.
[b] Samples obtained during first week of disease from patients with fatal infections.

TABLE 6. *Types of serological reactions[a] in 94 bacteriologically verified cases of various forms of melioidosis*

Type of infection	No. of patients[b]				
	Total	IHA+ CF+	IHA+ CFV	IHAV CF+	IHAV CFV
Localized pulmonary	53	46	2	4	1
Pulmonary and other organ	9	8		1	
Localized wound	16	10	1	5	
Wound and other organ	7	5	1	1	
Septicemia	5	4		1	
Other	3	2	1		
Unknown	1	1			
Totals	94	76	5	12	1

[a] Eight days through 6 months after disease onset.
[b] Symbols: +, positive; V, variable negative and positive.

seronegative patients died during the first week of disease. Three patients were positive by CF test only, and one was positive by IHA test only. However, only single serum samples were available from three of four patients with discrepant IHA and CF test results. The serological findings with the two tests on 455 sera from bacteriologically proved cases correlated 87%.

The 59 sera with noncorrelative reactions were from 23 patients. Thirty-four of 59 samples were obtained from 8 days to 6 months after disease onset from 18 patients. Approximately half of the noncorrelative sera obtained during this time interval were marginally reactive, viz., partial but less than 50% fixation at 1:4 dilution on CF test or reactive at 1:20 dilution on the IHA test. Sera, which were negative or marginally reactive on either IHA or CF tests, were respectively selected from three patients each and retested with homologous antigens prepared from isolates derived from the patients. Test results with homologous antigen were the same as that obtained with regular test antigens.

The distribution, according to type of disease, of different serological reactions in patients could be tabulated for 94 of the bacteriologically proved cases (Table 6). Inconsistent serological reactions of 12 of 18 patients in this category were manifested with IHA test, 5 with CF test, and 1 with both tests. Variable IHA reactions were found most frequently (5 of 16 cases) in patients with localized wound or burn infections and were also seen in approximately 10% of the patients with localized pulmonary disease. There appeared to be no selective distribution of CF variable reactions according to type of infection.

Information on time of disease onset and time of collection of sera was available for 169 patients, of which 108 had culturally confirmed infections. All were tested for antibodies with IHA test and all but 5 for antibodies with the CF test. The distributions of IHA titers of 445 sera from 169 cases and of CF titers of 401 sera from 164 cases by time after disease onset are shown in Tables 7 and 8, respectively. When more than one serum from a patient was tested in a specified time interval, findings on only one of the specimens were tabulated. Differences in titer of such multiple specimens usually were not greater than twofold. Positive IHA and CF reactions, frequently at high titer, were seen in 38 to 71% of samples obtained during the first week of disease. Within time intervals from 2 weeks to 9 months after disease onset, detectable antibodies were demonstrated by IHA tests in 88 to 100%, and by CF tests in 93 to 100% of the samples. High titers were usually attained by the third week of disease. Titers persisted for 9 months or longer. In this series, peak geometric mean IHA titer of 1:816 and CF titer of 1:99 were seen during the 4th to 5th month after disease onset.

Thirty-one patients provided samples 9 months or more after disease onset. *P. pseudomallei* was isolated from all but 5 of 31 patients. Persistence of antibodies at various time intervals after 9 months was demonstrated in 80 to 100% of the samples with IHA and CF tests. The data may have been biased by the disproportionately higher representation of patients (20 of 31) who had prolonged or persisting infections of 6 or more months duration. Nine patients in this late time series had responded well to antibiotic treatment

TABLE 7. *Distribution of HA titers of 445 sera from 169 cases of melioidosis by time of disease*

Time after disease onset	No. of sera with IHA titers (reciprocals)										Total	Positive 1:40 and greater	Reciprocal geometric mean titer
	Negative	20	40	80	160	320	640	1,280	2,560	> 2,560[a]			
												%	
2–4 days	9	2	1	1	3			3		1	20	45	19
5–7 days	4	4	1	1		2		1			13	38	20
8–14 days	1	2	5	5	3	1	3	3		2	25	88	159
15–21 days			5	1	6	4	1	3	4	2	26	100	356
22–28 days		3	3	4	1		6	7	1	3	28	89	362
29–60 days	2	2	2	5	13	12	12	18	4	4	74	95	410
61–90 days	2	2	2	10	7	12	8	4	6	5	58	93	321
91–120 days	3	2	3	3	2	7	5	12	4	5	46	89	395
121–150 days			3	2	2	4	9	3	7	7	37	100	816
151–180 days	2	1	2	1	1	7	3	3	9	5	34	91	559
181–270 days	2	2		5	6	7	4	3	3	10	42	90	470
271–365 days	1	1			2	4	1	3		3	15	87	416
1–2 yr		1	2	1	2	4	3	2	1	2	18	94	373
>2 yr	1	1			4	2	1	1			10	80	127

[a] Titers 1:5,120 through 1:20,480.

TABLE 8. *Distribution of CF titers of 401 sera from 164 cases of melioidosis by time of disease*

Time after disease onset	No. of sera with CF titers (reciprocals)									Total	Positive	Reciprocal geometric mean titer
	Negative	4	8	16	32	64	128	256	> 256[a]			
											%	
2–4 days	6			4		1			2	13	54	8
5–7 days	2		1	1		1	1	1		7	71	16
8–14 days			2	5		3	1	4	3	18	100	69
15–21 days	1	1	2	5		3	2	4	4	22	95	58
22–28 days		1	1	2	3	4	5	2	5	23	100	87
29–60 days	4			12	7	4	18	10	13	68	94	80
61–90 days	4	1	3	6	4	10	18	4	7	57	93	60
91–120 days	3		1	3	6	9	7	9	6	44	93	75
121–150 days	1		2	3	3	5	8	5	8	35	97	99
151–180 days	1			5	4	6	5	7	4	32	97	81
181–270 days	2			5	4	11	5	8	7	42	95	83
271–365 days			3	1	1	2	2	4	1	14	100	67
1–2 yr	1			2	4	6	3		1	17	94	46
>2 yr			1	1	3		2	2	1	10	100	69

[a] Titers 1:512 through 1:1,024.

and had a relatively limited clinical course of disease or infection. The disease history on two patients was incomplete. The late serological findings in patients with limited and prolonged infections are summarized (Table 9). Antibodies were detectable by both tests in all but three patients. The three exceptions had variable positive and negative test reactions during their course of disease. Within the limitations of the number of patients tested, the geometric mean IHA and CF titers of patients with prolonged infection were approximately 1.5 to 3-fold higher than those of patients with a limited disease course. Four of seven patients with histories of prolonged disease had recurrent episodes of disease at the time specimens were taken, two or more years after disease onset. A sample from one of the four patients was obtained 7 years after initial disease. The duration of the positive serological reactions could not be related to the type of disease. Persistent high-titer reactions were seen in patients with subacute disease of

TABLE 9. *Persistence of IHA and CF antibody titers in patients with a limited or prolonged course of infection*[a]

Time after disease onset	No. of patients positive/no. tested (GM titer)			
	Limited infection		Prolonged infection	
	IHA	CF	IHA	CF
9–12 months	3/4 (269)	4/4 (54)	9/10 (442)	9/9 (119)
1– 2 yr	6/6 (254)	5/6 (20)	10/11 (267)	10/11 (50)
>2 yr	2/3 (59)[b]	3/3 (40)[c]	6/7 (177)	7/7 (86)

[a] Patients with limited infection responded well to antibiotic treatment and were free of signs of infection within a 6-month period. The duration of signs of disease or infection in prolonged infections was 6 months or longer.

[b] Times of sampling: 2 years, 1 month (IHA-negative); 2 years, 8 months; 3 years, 2 months.

[c] Time of sampling: 2 to 2.5 years, three patients (one IHA test negative); 3 to 3.5 years, three patients; 7 years, one patient (chronic case).

several weeks to several months duration, as well as in patients with prolonged complicated infections.

DISCUSSION

A variety of serological procedures have been proposed or utilized for the serological diagnosis of melioidosis. From various published reports (2, 4, 5, 12), conventional agglutination tests with fixed bacterial cells, CF tests utilizing trichloroacetic acid extracts of organisms as antigen, IHA tests conducted with protein extracts, and skin tests with melioidin antigens had limitations in sensitivity or specificity, or both. On the other hand, IHA procedures conducted with Boivin (5), polysaccharide (12), or culture filtrate antigens (8, 10), and CF tests conducted with sonically treated (12) or aqueous extracts of organisms (2, 10, 11) appeared to be promising serological diagnostic tools. The factors which led to our selection of the IHA test of Ileri (8) and the described CF test were safety and simplicity in preparation of antigens, the ready applicability of the tests by diagnostic laboratories, and favorable preliminary findings on sensitivity and specificity of the tests. Minor modifications of the IHA test were made to improve the standardization of reagents.

In our experience, the described IHA and CF tests were valuable tools for diagnosis of human cases of melioidosis. The IHA and CF tests served to demonstrate significant antibodies, respectively, in 97% of 112 and 99% of 117 culturally proved cases when paired or serial serum samples were tested. In approximately 19% of 94 culturally proved cases, transient seronegative reactions during the course of disease were seen with either IHA of CF tests, but rarely with both tests. Negative and low-titer reactions in proved cases could not be related to possible antigenic differences among strains. Variable negative and positive serological reactions occurred more frequently with the IHA procedure and were demonstrated more often in cases with localized forms of disease. Similar discrepant serological findings in human melioidosis were also reported by Fournier et al. (5).

The CF and IHA test findings on 445 sera from 114 patients, from whom *P. pseudomallei* was isolated, correlated 87%. The CF test appeared to be slightly more sensitive. Both IHA and CF tests had excellent specificity when evaluated with sera from normal human beings. However, the high specificity of the CF test was not affirmed when tests were done with diverse antibacterial sera from hyperimmunized rabbits and from human patients. The CF antigen appeared to contain a common antigen with some but not all types of *P. aeruginosa*. In tests on nonmelioidosis patients, the CF test elicited reactions in sera from 5 of 11 patients with *P. aeruginosa* or *P. stutzeri* infections, but only in 7 sera from 96 patients with other diseases. Nonspecific reactions with IHA tests were found rarely with *P. aeruginosa* and other diverse antibacterial sera. Ten per cent of sera from patients with other diseases had reactions at low-titer levels of 1:20 to 1:40. Simultaneous "nonspecific" cross-reactions in IHA and CF tests were rare. If a titer of 1:8 in lieu of 1:4 is used as a criterion of a significant CF reaction (Table 8), the specificity of the procedure can be improved without any appreciable effect on its sensitivity.

The course of the serological response as determined by IHA and CF tests was similar. Antibodies were detectable in all but a few patients by the second week of disease. Significant rises in antibody titer during the early course of disease

were demonstrated infrequently. Moreover, in most such cases, titer rises were fortuitously demonstrated during the course of study of patients with fevers of undetermined origin. The paucity of demonstrable titer rises primarily reflects the fact that, in subacute melioidosis, patients are rarely seen when initial disease signs occur. The initial signs may be mild and easily disregarded. By the time acute symptoms develop and medical attention is sought, detectable antibodies are usually present. In some cases the disease was a recurrent manifestation of previously unrecognized latent or chronic melioidosis. The high percentage of positive IHA and CF reactions in sera obtained during the first week of disease is not surprising, since the purported time of disease in most of these cases was probably specious.

By the third week of disease, high antibody titers were usually present. Persistence of antibodies was demonstrated for a large number of patients who provided serial serum samples over a 6- to 9-month period after disease onset. In a relatively small number of patients tested, antibodies were detectable from 2 to 3.5 years, and in one case as long as 7 years, after infection. Available case-history data provided no evidence that serological response of patients had prognostic significance. Persistence of titers occurred in chronic cases as well as in cases with a limited disease course. The status of the patient's infection could not be related to antibody titer.

The observed persistence of antibodies in a large proportion of cases serves to affirm the potential usefulness of the IHA and CF procedures for seroepidemiological surveys of human populations. The IHA test has been used advantageously for diagnosis of melioidosis in goats and pigs. The CF test appears to have good sensitivity in detecting antibodies in experimentally infected monkeys (13). Its applicability to other animal species has not been established.

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