TABLE 1.
Ornithosis in Seashore Birds by Complement Fixation Test.

Species	No. Tested	No. AC	No. +	% +	Complement fixation serum titers 1:5	1:10	1:20	1:40	1:80	1:320
Laughing gull	60	3	24	40	10	8	1	2	2	1
Willet	37	3	17	45.4	7	6	4			
Skimmer	15	0	8	53	4	2	2			
Sanderling	8	1	4		4					
Royal tern	15	0	1	6			1			
Least tern	8	0	2			1		1		
Common tern	6	0	3		1	11				
Gull billed tern	6	0	1	16		1				
Ring billed gull	1	0	0							
Glossy ibis	1	0	1						1	
Brown pelican	4	1	0							
Sooty tern	1	0	0							
Reddish egret	3	3	0							
	165	11	61	36	26	20	8	3	3	1

complement with lymphogranuloma venereum antigen and with psittacosis antigen.

The significance of finding evidence of ornithosis in sea-shore birds serves to add to the spectrum of information regarding the distribution of this disease in nature. While it may not be a great hazard to the human population, since these sea-shore birds are not generally confined in close association with man, nevertheless, this disease appears to exist in a multitude of avian species. Under proper circumstances these infected birds might contribute directly, or through other birds, to the morbidity rate of human ornithosis.

Summary. Serological evidence of ornithosis-like infections among several species of sea-shore birds is presented. Over 40% of the serums of sea gulls, willets, and skimmers which were examined showed complement-fixing antibodies for ornithosis. One strain of an ornithosis-like agent has been isolated from the willet species.

15746

Specific Complement-Fixing Diagnostic Antigens for Colorado Tick Fever.

CARL J. DE BOER, LAWRENCE J. KUNZ, HILARY KOPROWSKI, AND HERALD R. COX.
From the Section of Viral and Rickettsial Research, Lederle Laboratories Division, American Cyanamid Company, Pearl River, New York.

In the past Colorado tick fever[1,2] was believed to be a mild form of Rocky Mountain spotted fever.[3] However, largely due to the work of Florio and his colleagues,[4,5] it is now known that Colorado tick fever is not a rickettsial infection but a viral disease,[5] presumably tick-borne,[6] which apparently is quite distinct from Rocky Mountain spotted fever[1,3,7] and dengue fever[8,9] although resembling the latter both clinically and hematologically.

1 Becker, F. E., *Colorado Med.*, 1930, **27**, 36, 87.

2 Toomey, N., *Ann. Int. Med.*, 1931-32, **5**, 585.

3 Florio, L., Mugrage, E. R., and Stewart, M. O., *Ann. Int. Med.*, 1946, **25**, 466.

4 Florio, L., Stewart, M. O., and Mugrage, E. R., *J. Exp. Med.*, 1944, **80**, 165.

5 Florio, L., Stewart, M. O., and Mugrage, E. R., *J. Exp. Med.*, 1946, **83**, 1.

6 Topping, N. H., Cullyford, J. S., and Davis, G. E., *Publ. Health Rep.*, 1940, **55**, 2224.

7 Shaffer, F. C., *Colorado Med.*, 1935, **32**, 226.

8 Florio, L. Hammon, W. McD., Laurent, A., and Stewart, M. O., *J. Exp. Med.*, 1946, **83**, 295.

In previous communications submitted from this laboratory[10-12] a method was described for preparing specific diagnostic antigens for certain rickettsial and neurotropic viral diseases. The adaptation by Koprowski and Cox[13,14] of Colorado tick fever virus to the brain tissue of the laboratory mouse provided means of obtaining large amounts of infectious material for the preparation of complement-fixing antigen. The present work deals with the application of the above cited method in the preparation of complement-fixing antigen for the diagnosis of Colorado tick fever.

Materials and Methods. Virus Strains. Three strains of Colorado tick fever virus—the Florio (Fl), the Baker (B), and the Condon (C)—originally obtained from Dr. Lloyd Florio,*,[4] and adapted to the mouse brain in this laboratory,[13,14] were used for the preparation of the complement-fixing antigen. The Fl strain had undergone 30 hamster[4] and 35 mouse brain passages.[13] The B strain had been carried through 5 hamster[4] and 10 mouse brain passages,[14] while the C strain was adapted directly from the serum of a naturally infected human case to the brain of the dilute brown agouti (dba) mouse,[14] following which it was carried through 10 brain-to-brain passages in Swiss albino mice.

Stable virus preparations were readily obtained by preparing 5 or 10% infected brain suspensions in a 50-50 mixture of normal rabbit serum-saline (pH 7.4) and storing in the dry ice chest.

Antigens. Three groups of 300, 21-day-old Swiss albino mice were injected intracerebrally with 0.03 ml of a 1:50 dilution of infectious mouse brain suspension of the Fl, B and C strains of virus, respectively. Ten percent normal rabbit serum in saline was used as diluent. Four days later the animals in all 3 groups showed nervous symptoms but relatively little paralysis. The brains were removed aseptically from all mice of each group and antigens were prepared by lyophilization followed by benzene extraction, as previously reported by De-Boer and Cox.[11,12] Prior to processing, each lot of infected mouse brain suspension was tested for infectivity by intracerebral inoculation in 21-28-day-old Swiss albino mice. LD_{50} titers ranging from 10^{-6} to approximately 10^{-7} were obtained.

Immune serum. The Fl strain was used for the production of all immune serum by hyperimmunizing mice. Swiss albino mice, approximately 2 months old, were injected intraperitoneally with 0.5 ml of a 1:50 dilution of infected mouse brain suspension in distilled water. Thereafter, the mice were reinjected at weekly intervals with 0.5 ml of a 10% brain suspension until tests conducted with serum obtained from trial bleedings indicated that a satisfactory antibody titer was secured. A total of 10 injections was given before the mice were exsanguinated and their sera used for the tests reported herein. In addition several serum samples from clinically diagnosed human cases of Colorado tick fever which occurred in Colorado, obtained through the courtesy of Drs. Lloyd Florio and H. L. Morency,† were subjected to the tests.

Complement-fixation Tests. Complement-fixation tests were carried out identically to those reported previously.[12] Antigens were titrated for antigenic activity in the presence of various dilutions of homologous immune sera. The dilution of antigen giving the highest titer for the immune serum was used

9 Pollard, M., Livesay, H. R., Wilson, D. J., and Woodland, J. C., PROC. SOC. EXP. BIOL. AND MED., 1946, **61**, 396.

10 Wolfe, D. M., Van der Scheer, J., Clancy, C. F., and Cox, H. R., *J. Bact.*, 1946, **51**, 247.

11 De Boer, C. J., and Cox, H. R., *J. Bact.*, 1946, **51**, 613.

12 De Boer, C. J., and Cox, H. R., *J. Immunol.*, 1947, **55**, 193.

13 Koprowski, H., and Cox, H. R., PROC. SOC. EXP. BIOL. AND MED., 1946, **62**, 320.

14 Koprowski, H., and Cox, H. R., in manuscript. Presented before the American Society of Tropical Medicine, Miami, Florida, November 5, 1946.

* Professor of Preventive Medicine and Public Health, School of Medicine and Hospitals, University of Colorado, Denver, Colo.

† City Health Officer, Boulder, Colo.

TABLE I. Titration of Colorado Tick Fever Antigen.

Antigen	Dilution	Immune mouse serum — Serum dilutions 1:4	1:8	1:16	1:32	1:64	1:128	1:256	Controls — Serum dilutions 1:4	1:8	1:16	Antigen	Red blood cells	Hemolytic system
Fl	1:1	4	4	4	4	2	0	0	0	0	0	0	4	0
	1:2	4	4	4	4	1	0	0				0		
	1:3	4	4	4	4	±	0	0				0		
	1:4	4	4	4	2	0	0	0				0		
	1:8	4	4	±	±	0	0	0				0		
B	1:1	4	4	4	4	0	0	0	0	0	0	0	4	0
	1:2	4	4	4	4	1	0	0				0		
	1:3	4	4	4	4	1	0	0				0		
	1:4	4	4	4	2	0	0	0				0		
	1:8	4	4	2	0	0	0	0				0		
C	1:1	4	4	4	4	0	0	0	0	0	0	0	4	0
	1:2	4	4	4	4	±	0	0				0		
	1:3	4	4	4	4	1	0	0				0		
	1:4	4	4	4	4	1	0	0				0		
	1:8	4	4	4	2	0	0	0				0		

4 = complete fixation of complement. 3, 2, 1 = proportionately lesser fixation. ± = trace. 0 = no fixation.

in subsequent complement-fixation tests. Although this may require more frequent rechecking of the titer of an antigen, it is of value in obtaining the optimal titers of unknown sera. Complement was always titrated in the presence of antigen thus diluted, increasing the amount of 1:30 dilution of complement by 0.025 ml instead of 0.05 ml, thereby obtaining a more precise and sensitive test.

Neutralization Test. Equal volumes of undiluted test serum and aliquots of serial 10-fold dilutions of mouse brain suspension infected with either Fl or C strains of Colorado tick fever virus were mixed, incubated in a water bath at 37°C for 2 hours, and inoculated into 21-28-day-old Swiss albino mice by the intracerebral route. Normal human serum was used as control.

Experimental. Titration of Colorado Tick Fever Antigens. Table I shows the results obtained when the antigens prepared from Fl, B and C strains were titrated in the presence of an immune mouse serum prepared with Fl strain. It is apparent that all 3 antigens gave approximately the same degree of fixation of complement with the Fl serum.

In numerous additional tests it was determined that the benzene extracted antigens prepared from each of the 3 strains of Colorado tick fever virus consistently gave negative results in the presence of highly positive Wassermann human sera. These results thus confirm and extend the observations previously reported by DeBoer and Cox[11,12] in that benzene-extracted antigens are highly specific and do not give false positive reactions in the presence of markedly positive syphilitic sera.

Cross-fixation Tests with Colorado Tick Fever Immune Serum and Heterologous Antigens. To check the antigenic identity of the virus, Colorado tick fever immune mouse serum was tested against a number of viral and rickettsial antigens by the complement-fixation reaction. The Eastern (EEE) and Western (WEE) equine encephalomyelitis antigens were derived from infected chick embryos; the murine (endemic) typhus,

TABLE II.
Complement-fixation Tests with Colorado Tick Fever Immune Serum and Various Viral and Rickettsial Antigens.

	CTF* immune mouse serum							Controls					
	Serum dilutions							Serum dilutions					
Antigen	1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:2	1:4	1:8	Antigen	Red blood cells	Hemolytic system
Western E.E.	0	0	0	0	0	0	0	0	0	0	0	4	0
Eastern E.E.	0	0	0	0	0	0	0				0	4	0
St. Louis	0	0	0	0	0	0	0				0	4	0
Japanese B	±	0	0	0	0	0	0				0	4	0
Rabies	0	0	0	0	0	0	0				0	4	0
Murine typhus	0	0	0	0	0	0	0				0	4	0
RMSF†	0	0	0	0	0	0	0				0	4	0
CTF*—Fl	4	4	4	4	4	2–	0				0	4	0
CTF*—B	4	4	4	4	4	2	0				0	4	0
CTF*—C	4	4	4	4	4	1	0				0	4	0
American Q	0	0	0	0	0	0	0				0	4	0

* CTF = Colorado tick fever. †RMSF = Rocky Mountain spotted fever.

TABLE III.
Complement-fixation Tests with Colorado Tick Fever Human Sera.

			Human sera							Controls						
			Serum dilutions							Serum dilutions						
Serum	Blood taken* months	Antigen	1:1	1:2	1:4	1:8	1:16	1:32	1:64	1:1	1:2	1:4	1:8	Antigen	Red blood cells	Hemolytic system
No. 1	33	CTF—Fl	±	0	0	0	0	0	0	0	0	0	0	0	4	4
No. 2	2½	CTF—Fl	4	4	4	4	±	0	0	4	2	0	0	0	4	0
No. 3	2½	CTF—Fl	4	4	4	±	0	0	0	4	4–	0	0	0	4	0
No. 4	42	CTF—Fl	±	0	0	0	0	0	0	0	0	0	0	0	4	0
No. 5	7	CTF—Fl	4	4	4	1	0	0	0	0	0	0	0	0	4	0
		RMSF	±	0	0	0	0	0	0					0	4	0
		Kolmer	0	0	0	0	0	0	0					0	4	0
No. 6	34	CTF—Fl	4	4	4	1	0	0	0	0	0	0	0	0	4	0
		CTF—B	4	4	4	0	0	0	0					0	4	0
		CTF—C	4	4	4	0	0	0	0					0	4	0
		RMSF	0	0	0	0	0	0	0					0	4	0
		Kolmer	0	0	0	0	0	0	0					0	4	0

* Counting from day when diagnosis of Colorado tick fever was made.

Rocky Mountain spotted fever (RMSF) and American Q fever antigens were prepared from infected yolk sacs; while the St. Louis and Japanese B encephalitis and rabies antigens originated from infected mouse brains.‡ The antigens fixed complement in the presence of their homologous antisera in the following dilutions: EEE 1:64; WEE 1:64; murine typhus 1:128; RMSF 1:16; American Q 1:1024; St. Louis 1:64; Japanese B 1:64, and rabies 1:128.

The results obtained in testing the above antigens in the presence of a Colorado tick fever immune mouse serum are shown in Table II. No fixation of complement was obtained with any of the heterologous antigens, although all 3 Colorado tick fever antigens showed good fixation titers.

Complement-fixation Tests with Convalescent Sera. Table III summarizes the results of complement-fixation tests with Colorado tick fever antigen and human sera obtained at different intervals after the onset of the disease.

Serum No. 1 was taken from a person who had suffered a natural infection 33 months previously. Sera No. 2 and No. 3 were taken 2½ months after experimentally induced infections and were stored in the frozen state for 42 months. Serum No. 4 was from the same person as No. 3 but taken 42 months after the original infection and 30 months after another injection of virulent material. Sera No. 5 and No. 6 were from persons who had had natural infections 7 and 34 months prior to bleeding, respectively. These sera were tested for complement-fixing antibodies in the presence of Colorado tick fever, Rocky Mountain spotted fever and Kolmer Wassermann antigens.

The results shown in Table III indicate that sera No. 1 and No. 4 taken 33 and 42 months respectively after an attack of the disease, demonstrated only traces of complement-fixing antibodies in the presence of Colorado tick fever antigen. Sera No. 2 and No. 3 taken 2½ months after diagnosis of illness, and No. 5 and No. 6 taken 7 and 34 months, respectively, apparently showed significant antibody titers. The specificity of the test is again demonstrated by the fact that sera No. 5 and No. 6 showed fixation only with Colorado tick fever antigen and not with Rocky Mountain spotted fever, nor Kolmer Wassermann antigens. In addition, it is seen that serum No. 5 fixed complement equally well in the presence of Colorado tick fever antigens Fl, B and C. This latter observation would seem to indicate that these 3 strains of Colorado tick fever are very similar antigenically, if not identical.

Comparison of Complement-fixing and Neutralizing Antibodies in Human Convalescent Sera. Table IV shows the results obtained in testing Colorado tick fever convalescent sera for their complement-fixing and neutralizing antibody titers. Since the serum samples were taken at various intervals from infected human cases, this test furnishes additional information regarding the time of appearance of complement-fixing or neutralizing antibodies and the length of time they persist during the convalescent period.

The results of the neutralization tests were in close agreement with those of the complement-fixation tests in all cases where the sera were not anticomplementary. Sera No. 2 and No. 3 were anticomplementary in dilution 1:1 and 1:2 to 2+ and 4+, respectively, which may account for the relatively higher complement-fixing titers (see Tables III and IV).

There is also close correlation in the results obtained with the 2 types of tests performed on sera of patients Ca, Og, Mo and Wi. For instance, the Ca serum, obtained 2 days after the diagnosis of Colorado tick fever, was devoid of all complement-fixing or neutralizing power. On the other hand, the Og serum taken on the 9th day after the disease was diagnosed, showed some complement-fixing and neutralizing capacity. Mo and Wi sera had definite complement-fixing power although their neutralizing titers were low. It may be added, that the results of

‡ All antigens, with the exception of American Q fever, were prepared by benzene extraction procedures. The American Q fever antigen was an ether extracted-washed rickettsial body preparation.

TABLE IV.

Comparison of Complement-fixation and Neutralization Tests with Colorado Tick Fever Human Convalescent Sera.

Serum	Blood* taken	Antigen	Complement-fixation tests† Serum dilutions 1:1	1:2	1:4	1:8	1:16	1:32	Neutralization tests‡ Mortality ratio of mice inoculated with virus dilutions 10-1	10-2	10-3	10-4	10-5	10-6	10-7	10-8	LD_{50} titer	Calculated Protective index§
No. 1	33 mos.	Fl	±	0	0	0	0	0	6/6	6/6	6/6	6/6	4/5	0/5	1/5		$10^{-5.50}$	4
No. 2‖,¶	2½ ''	Fl	4	4	4	4	±	0		6/6	3/5	4/5	0/6	0/5			$10^{-4.10}$	112
No. 3‖,¶	2½ ''	Fl	4	4	4	±	0	0		6/6	5/6	3/6	2/6	0/6			$10^{-4.15}$	100
No. 4‖	42 ''	Fl	±	0	0	0	0	0		6/6	6/6	3/6	0/5	0/5			$10^{-4.00}$	141
No. 5	7 ''	Fl	4	4	4	1	0	0	6/6	3/6	0/5	0/5					$10^{-2.00}$	14,130
No. 6	34 ''	Fl	4	4	4	1	0	0	6/6	4/6	1/6	1/6					$10^{-2.45}$	5,012
Ca	2 days	Fl	2–	0	0	0	0	0			6/6	6/6	5/6	1/6	0/6		$10^{-5.50}$	4
Og	9 ''	Fl	3	2	0	0	0	0			6/6	6/6	0/6	0/6			$10^{-4.50}$	45
Mo	14 ''	Fl		4	4	4	3	±			6/6	5/6	1/6	0/6			$10^{-4.50}$	45
		B		4	4	2	0	0										
		C		4	4	1	0	0										
Wi	18 ''	Fl		4	4	2	0	0			6/6	4/6	2/6	1/6			$10^{-4.60}$	35
		B		4	3	0	0	0										
		C		4	3	0	0	0										
Normal control												6/6	6/6	3/6	0/6	1/6	$10^{6.15}$	0
Su	2nd febrile rise	Fl		0	0	0	0	0			6/6	6/6	6/6	5/6	0/6		$10^{-6.40}$	0**
	22 days	B		4	4	4	0	0			6/6	6/6	4/6	0/4			$10^{-5.25}$	14**
		C		4	4	4	0	0										
	76 ''	B		4	4	3	0	0		6/6	5/6	1/4	0/6	1/6			$10^{-3.40}$	1,000**
		C		4	4	4	0	0										
		Kolmer		0	0	0	0	0										

* Counting from day when diagnosis of Colorado tick fever was made.
† Complement-fixation tests made by C.J.D. and L.J.K.
‡ Neutralization tests made by H.K.
§ Based on difference in LD_{50} titer from normal control serum.
‖ Induced experimental infection.
¶ Sera No. 2 and No. 3 were anticomplementary in dilutions 1:1 and 1:2 to 2+ and 4+ respectively
** Based on difference between convalescent and acute phase sera.

complement-fixation on the above sera were in close agreement regardless of whether the Fl, B or C antigens were used in the test.

The results of the tests with Sn sera are perhaps more illustrative than the above cited instances. Serum samples were obtained from the same individual at various intervals during and after illness. It may be observed that the Sn serum drawn during the second febrile rise was free from complement-fixing, as well as neutralizing antibodies. On the other hand, serum taken 22 days later showed positive complement-fixation in 1:8 dilution but neutralized only 14 LD_{50} doses of virus in mice. The complement-fixing antibody titer showed no significant difference between the 22nd and 76th days after diagnosis, but the neutralizing index of the serum increased from 14 to 1,000 during the same period. Again, the results of complement-fixation with the 3 samples of Sn serum were the same regardless of whether B or C antigens were employed in performing the test.

Summary and Conclusions. Specific diagnostic complement-fixing antigens for Colorado tick fever have been prepared from infected mouse brains. The benzene-extracted antigens employed gave no false positive reactions in the presence of highly positive human syphilitic sera. Cross fixation tests between Colorado tick fever immune serum and the heterologous antigens of viral and rickettsial origin indicate that Colorado tick fever is a distinct entity and the virus is not related to any of the other infectious agents tested. These results confirm those obtained with the mouse neutralization test previously reported.[13,14] Close correlation was obtained in the complement-fixation and mouse neutralization tests with human convalescent sera. The complement-fixing and neutralizing antibodies apparently appear in the blood of humans at about the 9th to 14th day after diagnosis of illness and may remain demonstrable as long as 34 months later.

The complement-fixation test, as well as the mouse neutralization test, may prove of value in epidemiological studies on the incidence and geographic distribution of Colorado tick fever. Furthermore, if results with animal sera parallel those obtained with human sera, the tests may be applied to study the ecology of Colorado tick fever.

15747

Studies on Bacterial Resistance to Streptomycin.*

CAROLINE A. CHANDLER AND EMANUEL B. SCHOENBACH. (Introduced by P. H. Long.)

From the Department of Preventive Medicine, The Johns Hopkins University School of Medicine, Baltimore, Md.

The rapid development of resistance to streptomycin by various organisms, both *in vitro* and clinically, has recently been reported by a number of workers.[1-4] Miller and Bohnhoff[1] have shown that gonococci and meningococci, originally susceptible to 8-40 units per cc can become resistant to concentrations of streptomycin as high as 75,000 units per cc within 4 to 6 transfers. Klein and Kimmelman[2,3] found that with the 12 strains of Shigella studied, resistance was increased from concentrations of 3-7 units to

* These investigations were supported by grants from Abbott Laboratories, Eli Lilly and Company, Lederle Laboratories, Inc., Parke, Davis and Company, and the Upjohn Company.

1 Miller, C. P., and Bohnhoff, M., *J. A. M. A.*, 1946, **130**, 485.

2 Klein, M., and Kimmelman, L. J., *J. Bact.*, 1946, **51**, 581.

3 Klein, M., and Kimmelman, L. J., *J. Bact.*, 1946, **52**, 471.

4 Alexander, H. E., *J. Pediatrics*, 1946, **29**, 192.