Enterovirus Typing by Complement Fixation. (29071)

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Complement fixation has been successfully employed in identifying unknown enteroviruses by their reactions with poliomyelitis(1-3), ECHO Types 1-14(4-6), and Coxsackie Group B, Type 1(5) antisera. This report extends the experience to identifications with specific monkey antisera of ECHO Types 15-25, Coxsackie Group B, Types 2-5, and Group A, Type 9. One hundred and forty-eight freshly isolated enterovirus samples grown in monkey kidney tissue culture were classified and the results correlated with neutralization tests. The diagnostic reliability and efficiency of a simple, 1-step complement-fixation technic using pooled ECHO antisera were demonstrated.

Materials and methods. Typing antisera were obtained from monkeys immunized respectively against poliomyelitis Types I-III,* ECHO Types 1-25,† Coxsackie Group B, Types 1-5,† and Coxsackie Group A, Type 9;† they were stored at —25°C. Just prior to testing they were heated 30 minutes at 60°C. When they proved anticomplementary, as did Coxsackie Group B, Types 1 and 2, Coxsackie Group A, Type 9, and ECHO Types 1-3, 5, 7, and 10, they were inactivated one hour undiluted, then diluted and reheated 30 minutes before use. When pools of the ECHO antisera were made, the anticomplementary specimens were distributed as equally as possible among them.

Viral antigens. Monkey kidney tissue cultures were maintained in tubes with 1.0 ml medium containing 85% 199H, 15% tryptose-phosphate broth, and antibiotics. At inoculation time the medium was replaced with 1 ml 10% or 20% fecal (or other) suspension, and the tubes incubated 90 minutes at 35°C. The inoculum was then replaced with 1 ml maintenance medium, and the cultures reincubated. They were observed daily for cytopathic effects, and the fluids harvested when cell degeneration was complete. One-tenth ml infected fluid was then inoculated into each of 3 tubes containing monkey kidney cells in 1 ml maintenance medium consisting of 400 mg glucose plus 2 ml calf serum added to 100 ml 80% 199H and 20% tryptose-phosphate broth. These cultures were incubated and the fluids harvested as soon as cell degeneration was complete. They were clarified by centrifuging 15 minutes in the cold (4°C) at 1000 *g*, and the undiluted, unheated supernates typed by complement fixation with monkey antisera.

Complement fixation. Following standard procedure(7,8) total volume was 0.5 ml, the reagents being used in 0.1 ml aliquots. The standard reagent diluent was 0.85% NaCl solution. In practice, 0.1 ml of optimally diluted antiserum was tested with 0.1 ml each of viral antigen and three 50% units (C'_{H50}) of complement (C'); 18-24 hours at 3°-6°C were allowed for fixation. Antisera and antigens were tested for nonspecific reactivity with uninoculated tissue culture fluid and with normal monkey serum, respectively. They were examined for anticomplementary activity with 1, 2 and 3 C'_{H50}, and C' stability was tested by incubating the same numbers of C'_{H50} alone; saline solution was substituted where necessary for omitted reagents. Following fixation, 0.2 ml optimally sensitized sheep erythrocytes (5×10^8 sensitized cells/ml) were added, and 30 minutes at 37°C allowed for hemolysis. Results were read by visual comparison with standards simulating percentages of hemolysis at 5% intervals from 0% to 100%. Reagents were considered not anticomplementary and C' stability satisfactory only if at least 85% and 100% hemolysis were observed with 2 and 3 C'_{H50}, respectively.

Results. In preliminary trials of sensitivity

* Obtained from Communicable Disease Center, U.S.P.H.S., Chamblee, Ga.

† Obtained from National Foundation, New York.

and specificity, each of the 34 typing antisera was tested by complement fixation with each of the corresponding antigens except ECHO Types 14, 21, and 23, which were not available. The antigens were used at the highest dilution found to yield complete fixation in preliminary titrations with serially diluted homologous antiserum. As additional tests of specificity, they were examined also with antigens of ECHO Types 26 and 27, and Coxsackie Group B, Type 6. At the optimal dilution, which was 1:256 for the majority of antisera, reactivity was restricted to the homologous antigen. ECHO antisera Types 17-20 were used at a 1:32 dilution for sensitivity, but specificity at this dilution was equally satisfactory. None of the antisera, when optimally diluted, showed evidence of anticomplementary activity or nonspecific reactivity with uninoculated tissue culture fluid.

In the early work, serologic identifications were accomplished by testing unknown viruses simply, by a 1-tube complement-fixation test, with each of the 34 antisera (Method I). Typing was completed 24 hours after cytopathic effect was complete, but considerable work was involved. Later, pools of the poliomyelitis, the Coxsackie, and 5 pools of the ECHO antisera were used in screening, and the viruses then identified by retesting with individual components of the reactive pool (Method II); this decreased technical effort, but increased typing time from 24 to 48 hours. More recently in the case of ECHO antisera, 5 additional pools were prepared, each differing from the other 9 in all but one antiserum component. Testing synchronously with the 10 pools (Method III) made it possible to identify ECHO viruses simply within a 24-hour period. Similar technics have been applied in differentiating viruses by neutralization(9,10). Table I illustrates the manner of pooling antisera for Method III, and gives results obtained in a preliminary evaluation of specificity using standard ECHO antigens.

The reliability of complement fixation in identifying unknown virus isolates is illustrated in Table II; 148 viruses were typed,

TABLE I. Complement-fixation Tests with Pooled ECHO Antisera and Standard ECHO Antigens.

Pool No.	Antiserum types in pool	Result* with antigen type																							
		1	2	3	4	5	6	7	8	9	10	11	12	13	15	16	17	18	19	20	22	24	25	Normal
1	1,2,4,6,8	R	R	—	R	—	R	—	R	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
2	3,5,9,11,12	—	—	R	—	R	—	—	—	R	—	R	R	—	—	R	—	—	—	—	—	—	—	—
3	7,10,14,15,16	—	—	—	—	—	—	R	—	—	R	—	—	R	—	R	—	—	—	—	—	—	—	—
4	13,17,18,19,20	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	R	R	R	R	R	R	R	—
5	21,22,23,24,25	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	R	R	R	—
6	1,9,13,16,21	R								R				R		R								—
7	2,7,11,17,22		R					R				R					R				R			—
8	4,10,12,18,23				R						NT		NT					NT				R			—
9	3,6,14,19,24			R			R								R				R				R	—
10	5,8,15,20,25					R			R											R				—
	Normal serum	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—

* R = Reactive; — = Nonreactive; NT = not tested.

The pools were nonreactive also with antigens of ECHO Types 26 and 27, Coxsackie Groups A Type 9 and B Types 1-5, and poliomyelitis Types I-III. Pools 6-10 were composed of the same individual antisera as Pools 1-5 and were accordingly not retested for reactivity with heterologous antigens; economy of critical reagents was also a consideration. Pool 8 could not be tested with 3 of the corresponding standard antigens because supplies of the latter had been exhausted.

TABLE II. Correlation Between Complement Fixation and Neutralization Tests of Unknown Viruses.

Identity as established by Neutralization Type	No.	Complement fixation Unidentified	Identified by method* I	II	III
Coxsackie B_2	9		4	5	
" B_3	2		1	1	
" B_4	8			8	
" B_5	34		4	30	
" A_9	1		1		
ECHO 1	3		2		1
" 4	5		1	3	1
" 6	2			1	1
" 7	1				1
" 8	2	2			
" 9	4	1	2	1	
" 11	6	1	3	1	1
" 14	2		2		
" 17	3			2	1
" 20	1			1	
" 22	1				1
" 24	1				1
" 25	5		3		2
Polio I	50	2	35	13	
" III	8		3	5	
Total	148	6	61	71	10

* For detail of methods see Results paragraph 2.

using Methods I, II, and III as they developed; the neutralization test served as standard of reference. The complement-fixation and neutralization tests agreed in 95.9% of cases. Six (4.1%) of the unknown viruses were not definitively identified. The initial supply of monkey antisera, while generous, was insufficient to allow repeating the complement-fixation tests in these 6 cases. Possibly more precise determination of the optimal antiserum dilutions would have resolved some of the differences.

Discussion. The present study extends the work of other investigators who have successfully employed complement fixation to identify unknown enteroviruses. LeBouvier *et al* (1), Schmidt and Lennette(2), and Casey and Marchetti(3) identified only poliomyelitis isolates. These authors used both monkey kidney and HeLa cell tissue cultures for growing viruses and monkey hyperimmune sera for typing. The number of specimens examined and the percentages identified are comparable to those reported here. Halonen *et al*(4) used ECHO immune monkey sera types 1-14 to identify successfully 90% of 37 ECHO virus isolates grown in monkey kidney tissue culture. Godtfredsen(5) used poliomyelitis Types I, II, and III, ECHO Type 1, and a Coxsackie Group B, Type 1, antisera to type 58 of 71 isolates. Yohn and Hammon(6) dealt only with ECHO Type 4. In the present study 54 Coxsackie, 32 ECHO, and 56 poliomyelitis viruses were identified by complement fixation; 6 were not.

Summary. The results of complement-fixation tests using pooled and individual antisera of poliomyelitis Types I-III, ECHO Types 1-25, and Coxsackie Groups A Type 9, and B Types 1-5, agreed with viral neutralization tests in identifying 142 (95.9%) of 148 enteroviruses isolated by monkey kidney tissue culture.

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