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Quantitation of Enterovirus 70 Antibody by Microneutralization Test and Comparison with Standard Neutralization, Hemagglutination Inhibition, and Complement Fixation Tests with Different Virus Strains

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We describe here a microneutralization procedure for conveniently testing large numbers of specimens for antibodies to enterovirus 70. The test utilized human rhabdomyosarcoma cells and was read by staining with crystal violet after 4 days of incubation. The test compares well with other serological assays, being more sensitive than the standard tube neutralization test and the complement fixation test, but less sensitive than the hemagglutination inhibition test. However, the hemagglutination inhibition test required concentrated, partially purified virus as antigen, as did the complement fixation test, and was difficult to read, so that its greater sensitivity may not be of practical significance. By all four test procedures, a recent isolate of enterovirus 70 was a more sensitive antigen than the prototype strain, as shown by greater geometric mean titers in sera of patients from various epidemics.

Acute hemorrhagic conjunctivitis (AHC) is a new form of human eye disease that first appeared in Africa and Asia in 1969 and has caused major epidemics in 1970 to 1972 (10, 13, 15, 17, 26) and 1980 to 1982 (6, 9, 12, 23, 25). The disease is striking not only in its clinical presentation but also in its geographic limitation (to humid, coastal areas in the equatorial belt) and in its viral etiology. These aspects have recently been reviewed in detail (9).

The major etiological agent of AHC is a new picornavirus, enterovirus type 70 (EV70), which may have evolved from domestic animals in 1969 (16, 18, 19, 21, 24, 28) and which shares few antigenic relationships with other human enteroviruses (5, 17, 22). Serum neutralization (SN) tests in tubes of susceptible cell cultures have been the standard tests for identifying virus isolates and for conducting serological surveys for specific antibody in human sera, as is the case for other enteroviruses.

In the 1980 to 1982 pandemic of AHC, caused by EV70, the virus was unusually difficult to isolate and had, in fact, changed slightly in its genomic characteristics (9, 14). Most outbreaks were therefore identified solely by serological means. As the demand for serological testing increased, it was clear we needed additional tests which could handle larger numbers of serum specimens in each run. Accordingly, we developed a microneutralization test that is read by staining at 4 days, and we compared the serum titers with those obtained by standard tube SN, hemagglutination inhibition (HI), and complement fixation (CF) tests. In this report, we describe the micro-SN test procedure, using the prototype J670/71 strain and a current 1981 isolate as antigens to cover antigenic changes which appeared to have occurred between the 1970 to 1972 and 1980 to 1982 epidemics (M. H. Hatch, manuscript in preparation).

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MATERIALS AND METHODS

Virus production. Many strains of EV70 from eye swab specimens were used in this study, and two strains were used consistently for the serological tests: the prototype J670/71 from Hokkaido, Japan, in December 1971 (17), and V1250, the first Western Hemisphere isolate, from La Ceiba, Honduras, in August 1981 (9). Unless indicated otherwise, the viruses were propagated in human embryonic lung diploid fibroblast (HELF) cells and in human rhabdomyosarcoma (RD) cells, using Eagle minimal essential medium with 2% fetal calf serum as described previously (2, 7), except that the inoculum was routinely adsorbed for 1 h at 23°C and cultures were incubated in stationary position at 33°C (28). Cultures were harvested by three freeze-thaw cycles when all cells were visibly affected (4^+ cytopathic effect), usually at 48 h postinfection.

Sera. Human serum specimens were collected as acute-phase (S_1) and convalescent-phase (S_2) pairs from EV70-related AHC patients in Taiwan in 1971 (26), in Guam during the 1975 resettling from Vietnam (1), and in Thailand in 1980 (2). The serum pairs tested were randomly chosen from those collected in the initial studies and were not preselected for antibody titer or for appropriate spacing between the acute-phase and convalescent-phase sera. Sera were initially diluted 1:8 in growth medium (for SN tests), phosphate-buffered saline (PBS), pH 7.2 (for HI tests), or veronal-gelatin buffer (for CF tests) and heat inactivated at 56°C for 30 min before use in the serological tests.

Tube neutralization test. Standard tube SN tests were performed in HELF cells with serial twofold dilutions of heat-inactivated (56°C, 30 min) sera versus 30 to 90 50% tissue culture infective doses ($TCID_{50}$) of virus per 0.1 ml. Virus dose was determined by prior infectivity titrations in which 0.1 ml of serial twofold dilutions of virus was inoculated onto HELF cells in triplicate and adsorbed for 1 h at 23°C, and the cells were maintained under 1 ml of 98% Eagle

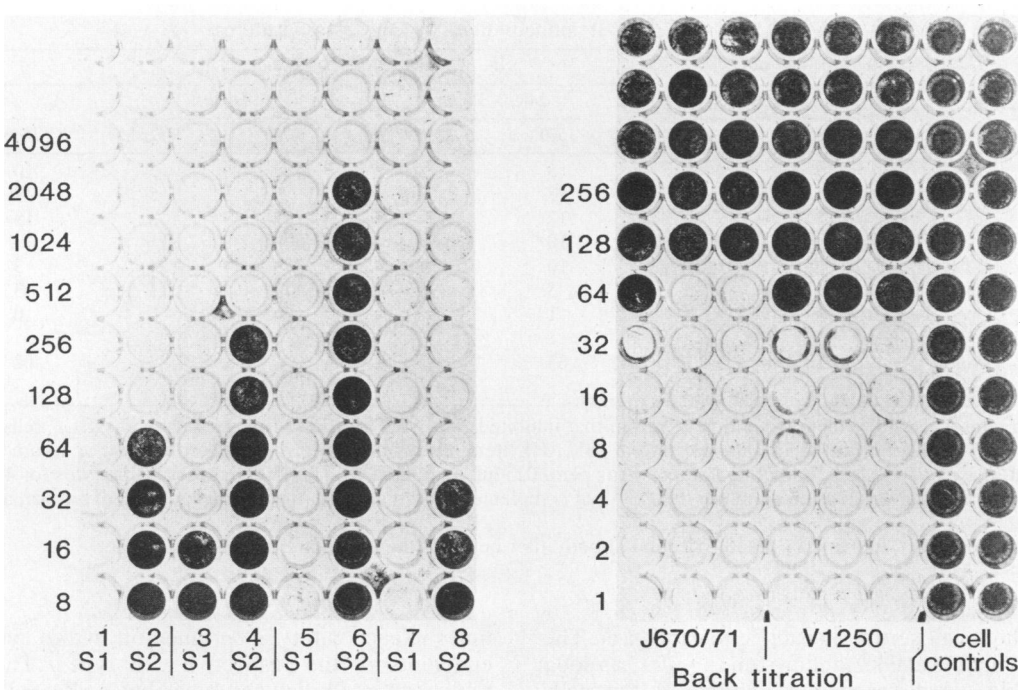

FIG. 1. A typical EV70 micro-SN test (left) with serial dilutions of four pairs of human sera versus V1250 at 32 $TCID_{50}$ per 0.05 ml; convalescent serum titers range from 1:32 (row 8) to 1:2,048 (row 6). The back titration and cell control plate (right) shows solid staining with the uninfected cells and back titrations of 53 and 32 $TCID_{50}$ per 0.05 ml for J670/71 and V1250, respectively.

minimal essential medium–2% fetal calf serum at 33°C for 14 days. The SN test likewise employed triplicate tubes (but with 0.2 ml of inoculum), an adsorption period, and the same maintenance and incubation conditions except that the test was terminated at 7 days.

Microneutralization test. The micro-SN test was adapted from one previously described for adenoviruses (8). Many parameters were evaluated during the development of the test, but for the sake of brevity only the final test procedure is described here. Sterile, 96-well, rigid, flat-bottom plates with plastic covers (no. 3596; Costar, Cambridge, Mass.) were used for the infectivity titrations and SN test. All reactants were dropped into the wells with 0.05-ml sterile disposable pipette droppers (no. 1-010-4101; Dynatech Corp., Alexandria, Va.). Diluting was done primarily with an Automatic Diluter (model 222-20-PU; Cooke Engineering Co. [Dynatech Corp.], Alexandria, Va.) equipped with 0.05-ml flame-sterilized loops or was done manually with a Titertek Finnpipette (no. 77-889-00; Flow Laboratories, Inc., McLean, Va.) with eight autoclaved polypropylene disposable microliter pipette tips (no. 9025; Medical Laboratory Automation, Inc., Mount Vernon, N.Y.). Use of the Automatic Diluter required adjustment of the moving platform so that the loops did not touch the bottom of the wells. For diluting, the loops were prewet in a tray of growth medium (GM) and then blotted with a sterile blotter. The diluent used throughout the microtest was the GM (90% Eagle minimal essential medium–10% fetal calf serum).

For microinfectivity titrations, 0.05 ml of GM was dropped into all wells except the first wells across the plate, 0.10 ml of virus stock was dropped into the first wells in up to seven replicas, and the virus was diluted in a twofold series. Generally, the virus stock was prediluted to 10^{-3} or 10^{-4} so that the endpoint dilution would be reached on the plate. Each plate contained a row of cell controls consisting of 0.10 ml of GM in the first well, which was diluted along with the virus. Then, 0.05 ml of GM was added to all wells in the plate, followed by 0.10 ml of a suspension of RD cells containing ca. 17,500 cells per 0.10 ml. The plates were incubated under 5% CO_2 at 36°C for 4 days. The cell control wells were examined for monolayer formation, and the plates were then stained with a vital stain. The staining procedure involved removing the culture fluid, immersing the plate in a fixative-stain solution containing 5.55% formaldehyde and 0.07% ethanolic crystal violet in 0.005 M PBS (pH 6.5) for 20 min, rinsing well in running water, and inverting on a towel to air dry (8). Virus titers were defined as the highest dilution of virus showing 0 to 1^+ staining as compared with the cell controls (4^+ staining) in 4 days; this dilution was considered to be 1 $TCID_{50}$ of virus per 0.05 ml.

For the micro-SN test, 0.05 ml of GM was dropped into all wells except the first row across, 0.10 ml of heat-inactivated serum at a 1:8 starting dilution was dropped into the first wells, and the serum was diluted in a twofold series. Then, 0.05 ml of a 30- to 90-$TCID_{50}$ dilution of virus was dropped into the wells, and the plates were incubated for 1 to 2 h at 23°C. During this time, a virus back titration was set up in triplicate, using the working virus dilution as the starting dilution; cell control wells were again included as in the microinfectivity titrations. The final step was the addition of 0.10 ml of RD cell suspension, incubation for 4 days, and staining as described above. A typical micro-SN test with its virus back titration is shown in Fig. 1.

Virus purification and concentration. HELF monolayers in 150-cm^2 flasks (T-150; Corning Glass Works, Corning, N.Y.) were drained of growth medium and inoculated with 2 ml of seed virus culture (multiplicity of infection = 1), the pH of which was adjusted with filter-sterilized 0.05 M acetic acid to reduce excess alkalinity. The virus was adsorbed for 1 h at 23°C, the inoculum was removed with a pipette, and 30 ml of

TABLE 1. Detection of antibody titers by four test procedures

Rise in antibody titer[a]	No. of serum pairs by test and antigen							
	Tube SN test		Micro-SN test		HI test		CF test	
	J670	V1250	J670	V1250	J670	V1250	J670	V1250
None	6	4	5	4	4	3	10	2
2-fold	16	11	17	11	8	5	13	14
4-fold	17	17	12	14	8	8	18	22
8-fold	10	11	10	10	10	11	13	9
16-fold	6	9	10	6	13	14	3	6
32-fold	5	5	5	7	11	13	3	5
≥64-fold	0	3	1	8	6	6	0	2
% of pairs with diagnostic rise[b]	63	75	63	75	80	87	62	73

[a] SN titers are reciprocals of the highest dilution of serum that inhibited 30 to 90 $TCID_{50}$ of virus for 7 days in HELF cells (tube test) or 4 days in RD cells (micro test), both in stationary position at 33°C. HI titers are reciprocals of the highest dilution of serum that completely inhibited the agglutination of 4 hemagglutinating units of virus per 0.025 ml, incubated with 0.4% human O erythrocytes for 4 h at 4°C. CF titers are reciprocals of the highest dilution of serum fixing 5 U of complement overnight with the optimal dilution of antigen as determined in block titrations.

[b] A diagnostic response is a fourfold or greater rise in antibody titer between the acute and convalescent sera.

medium 199 without calf serum was added to each flask. The cultures were incubated at 33°C and frozen at −70°C as soon as the cytopathic effect reached 4^+ (100% of the cells infected), usually 3 to 4 days postinfection. The cultures were frozen and thawed three times and clarified at 1,000 × *g* for 30 min at 4°C. To the supernatant fluid, NaCl and PEG 6000 were added to 2.3 and 10% final concentrations, respectively, in an ice bath and left overnight in ice to precipitate the virus. The precipitate was recovered and washed three times with 0.01 M PBS (pH 7.2) by centrifugation at 1,000 × *g* for 2 h at 4°C.

The virus was then further concentrated by centrifugation over a cushion of 1 ml of 70% sucrose in PBS and 1 ml of glycerol in SW41 tubes at 140,000 × *g* for 4 h at 20°C. The supernatant fluid was discarded, and the cushion containing the virus was collected and dialyzed against PBS in an ice bath.

Hemagglutination and HI tests. Hemagglutination tests were carried out with concentrated virus preparations, using 0.01 M PBS diluent (pH 7.2) and 0.5% avian and 0.4% mammalian erythrocytes according to standardized procedures (11), except that the tests were incubated for 4 to 16 h at 4°C as recommended by Kono et al. (20). For HI tests, sera of patients at the 1:8 starting dilution were adsorbed with 50% human O cells for 1 h at 4°C (11). Human O cells were used as indicator, and the tests were read after a minimum of 4 h at 4°C.

CF tests. CF tests were carried out with heat-inactivated sera of patients and overnight fixation of 5 U of complement according to standardized procedures (3). The antigen was the concentrated virus preparation described above. The optimal dilution of antigen for use in the test was predetermined by block titrations against serial dilutions of three representative EV70-convalescent sera.

RESULTS

Because EV70 was isolated in many different cell types, especially between 1970 and 1976 (9, 10, 15, 27), we first sought to establish the optimum cell culture for use in a 4-day micro-SN test. Nine cell types were chosen (ChangC, HeLa, HEK, HEp-2, pMK, Vero, HELF, RD, and MA104). Both the prototype and Honduran strains were passed four times in each cell type and then titrated to cytopathic effect endpoints in the respective cell types. The two strains replicated in all nine cell types but replicated to the highest titers in RD cells. Thus, RD cells were selected for the micro-SN test.

Antigenic differences among EV70 strains were suggested by the results of SN tests carried out to identify strains isolated between 1971 and 1981. All strains isolated between 1971 and 1975 were neutralized to homologous titer by a reference antiserum prepared against the J670/71 strain (5). Strains isolated from 1976 to 1981 were neutralized to significantly lower titers by this antiserum. Accordingly, we used two strains, J670/71 and V1250, in our serological evaluations of the four test systems (Table 1). The prototype J670/71 was representative of the pre-1976 group, and V1250 was representative of the post-1976 group. Both were used at 30 to 90 $TCID_{50}$ in the SN test, based on prior infectivity titrations. For the HI and CF tests, partially purified, concentrated virus, prepared as described above, was used. These antigens contained intact virions 28 nm in diameter (Fig. 2), 10^9 to 10^{10} $TCID_{50}$ of virus per ml, hemagglutination titers of 1:512 to 1:2,048 with human O erythrocytes at 4°C (1:128 to 1:1,024 with guinea pig erythrocytes and 1:1 to 1:2 with chicken erythrocytes at 4°C), and optimal CF antigen titers of 1:16 to 1:32.

The data are presented as the number of serum pairs exhibiting no change in titer, a twofold rise, a fourfold rise, etc. (Table 1). The micro-SN test was more sensitive than the tube SN test in that changes in titer detected were greater, although both tests had the same total number of positive serum pairs. The micro-SN test was also more sensitive than CF, both in numbers of seroconversions and in titers detected but was less sensitive than HI. However, the practical usefulness of the HI test is compromised by the need for a concentrated and preferably purified antigen. Additionally, sera tested by HI had to be adsorbed with erythrocytes to minimize the effect of nonspecific agglutinins, and the HI tests were difficult to read because of erratic back titrations. These findings are summarized as the percentage of serum pairs exhibiting a diagnostic rise in antibody titer. Again, the micro-SN test was comparable to the tube SN test, and both neutralization tests were more sensitive than CF and less sensitive than HI. The 1980

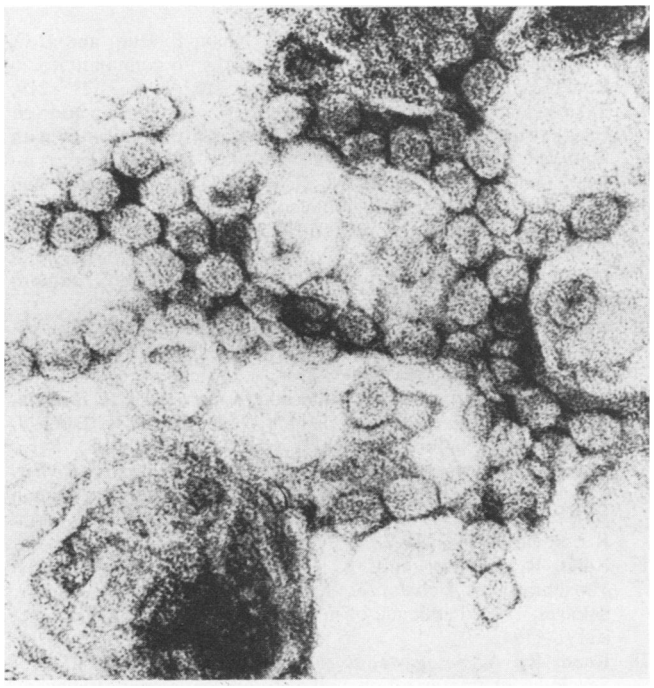

FIG. 2. Electron micrograph of a representative partially purified EV70 (V1250) preparation as used for the HI and CF tests; ×156,260.

isolate reacted better than the prototype strain in all test systems.

Finally, the three groups of sera were analyzed by geometric mean titers (GMTs) in all systems tested (Table 2). The GMTs are shown for each epidemic and also for the total. The results correspond well to those in Table 1. The HI test clearly detected the hightest titers of antibody, the CF test detected the lowest, and the micro-SN test was in between. The recent isolate was more sensitive in all systems.

DISCUSSION

Application of any serological test to large-scale serum surveys must usually consider strain variation in the preparation of the test antigen. We believed that to be particularly important in this study because we had already shown that there were significant differences in RNA oligonucleotide maps between early and recent EV70 strains (14). Further, EV70 isolates recovered before 1976 were neutralized to the homologous titer of a reference rabbit antiserum, whereas post-1976 isolates were neutralized to 4- to 16-fold-lower titers. Because these lines of evidence indicated genetic and antigenic changes in EV70 strains isolated between 1971 and 1981, we used both the prototype J670/71 and the recent V1250 strains of EV70 as antigens in the serological tests.

The development of the micro-SN test became necessary in 1981 when a new pandemic of EV70-related AHC was sweeping tropical, coastal areas of the world. In the Western Hemisphere, the disease began in Brazil in February 1981, spread along the northern coasts of South American by July, then spread throughout Central America, the Yucatan Peninsula, Key West and Miami, Florida, and the entire Caribbean Basin by October (9). Isolations of EV70 virus from these outbreaks were rare, although considerable effort was spent in the attempts (4, 6, 9, 23). Therefore, most of the outbreaks had to be etiologically documented by serological testing only.

The micro-SN test is convenient for any size test, but especially for large test runs. The use of disposable droppers and plates and an automatic diluting machine make the test even more practical because of the speed and ease with which it can be set up. Further, termination of the test after 4 days of incubation and reading of the titers by a simple staining of the cells make the test faster and less laborious than the standard tube SN test. Tube tests generally are incubated for 7 days before being read by degree of cytopathic effect in each tube, and each tube must be fed with fresh medium at 3 or 4 days to maintain pH. In terms of sensitivity, the micro-SN test gave the same total number of positive serum pairs as the tube test and gave greater changes in titer between acute and convalescent sera.

TABLE 2. Comparison of serological response of patients to EV70 infection by four test systems

EV70 epidemic	No. of serum pairs	Serum	GMT[a]							
			Tube SN		Micro-SN		HI		CF	
			J670	V1250	J670	V1250	J670	V1250	J670	V1250
Taiwan 1971	10	S_1	5.3	5.7	6.5	8.0	4.3	4.3	4.3	4.0
		S_2	17.1	22.6	13.0	24.3	42.2	59.7	9.8	16.0
Vietnam to Guam 1975	20	S_1	4.3	4.8	4.4	4.4	5.3	5.1	5.1	5.4
		S_2	21.1	26.0	24.4	26.7	56.3	57.7	23.4	26.0
Vietnam to Guam 1975 (matched pairs)[b]	20	Acute	5.7	6.7	6.5	8.0	4.4	4.6	5.3	6.1
		Convalescent	19.7	59.7	43.7	66.9	57.7	73.5	14.4	32.0
Cambodia to Thailand 1980	10	S_1	5.7	5.3	6.1	7.5	4.0	4.0	7.5	6.1
		S_2	48.5	48.5	36.8	60.3	55.3	68.6	45.3	45.3
Total	60	S_1	5.1	5.7	5.6	6.3	4.7	4.8	5.1	5.4
		S_2	22.9	37.2	28.6	40.9	53.9	64.7	19.3	28.2
Mean fold difference between S_1 and S_2			4.5	6.5	5.1	6.5	11.5	13.4	3.8	5.2

[a] GMT of endpoint dilutions, as defined in Table 1; a GMT of 4.0 is negative (<1:8).

[b] Age-, sex-, and city-matched serum pairs collected as single sera as the refugees left Guam for the United States.

Both the HI and CF tests are practical in serum surveys, but both have disadvantages that weigh against their routine application. The HI test in our hands was the most sensitive of the four tests evaluated, but it required prior absorption of the sera with erythrocytes and was often difficult to read; that is, the agglutination patterns were not always distinct. In a previous report, Kono et al. found HI titers with a purified virus preparation that closely paralleled SN titers rather than exceeding them (20); this may have been due to different approaches in treating the sera. The CF test, on the other hand, was as reproducible and easy to perform as CF tests for other respiratory and conjunctivitis viruses, but was the least sensitive of the four tests. Both tests require a concentrated preparation of purified or partially purified virus.

Thus, the micro-SN test, using a current EV70 strain as antigen, is clearly a test of choice for investigating EV70 epidemics. It combines the specificity of neutralization with the speed and convenience of a microtiter test and is easily read after 4 days of incubation by staining the uninfected cells.

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