Routine HA and HAI Tests for Identifying Enterovirus and Reovirus Strains

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IDENTIFICATION of enteroviruses by the neutralization test is costly and time consuming. There are 59 distinct types now recognized within this group (*1*). A number of "new" types with other distinct antigenic characteristics await official acceptance by the National Institutes of Health Panel for Enteroviruses (successor to the Committee on Enteroviruses). Still others await detection or description. Using monkey kidney cell tissue cultures (MKCTC), the most frequently employed cell system in laboratories doing routine enterovirus examinations, fewer than 40 of these types are isolated with any frequency. However, the task of identifying even this many types by the neutralization test is formidable.

Complement fixation tests for enterovirus typing have been proposed (*2*) and may prove useful. They are being studied in Communicable Disease Center laboratories, and their routine use will be discussed in future reports.

In 1957, it was reported (*3*) that ECHO types 3, 6, 7, 10 (now known as reovirus type 1), 11, and 12, and Coxsackie type B3 caused the agglutination of type O human erythrocytes under appropriate conditions, and that type-specific antiserums inhibited the effect. Studies which followed (*4–9*) showed that hemagglutination (HA) and hemagglutination inhibition (HAI) tests could be applied also to ECHO types 13, 19, and 21, Coxsackie types A7, A20, A21, A24, B1, and B5, and strain JV–10 (enterovirus 59). Hemagglutination by other enterovirus types has been reported from time to time but has not been confirmed. It also has been demonstrated that there may be special requirements for and limitations to these techniques: (*a*) only a limited number of strains of some virus types, particularly of ECHO 6 (*10*) and Coxsackie B1, B3, and B5 (*7*), cause an HA reaction; (*b*) optimal temperature for HA differs among the various virus types (*3*); (*c*) age of the erythrocyte donor may be of importance—fetal red blood cells produce higher HA titers than adult cells with Coxsackie B1, B3, and B5 (*7*); (*d*) virus strains may lose HA capacity when they are propagated in malignant "line" cells (*11*); (*e*) modifications of the media in which the tests are performed may enhance HA activity (reported by L. Rosen and J. Kern in a personal communication to the author).

The reovirus group (formerly ECHO 10) has been segregated into three distinct types largely on the basis of HAI antibody differences (*12*). Specific identification is therefore dependent on the use of the test that demonstrates these differences.

In the laboratories of the Enterovirus Unit, Communicable Disease Center, HA and HAI tests are now performed routinely as an early step in the identification of MKCTC isolates. This paper presents the results of tests performed on 666 unselected isolates during the past year.

Materials and Methods

The methods used are those of Rosen (*12*), with few modifications.

Viruses. All virus strains had been isolated

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in MKCTC from fecal samples or rectal swabs collected during 1960 and 1961 from healthy children or patients living in many different sections of the United States. Maximum HA titers were obtained when tissue culture tubes were incubated until all cells had been destroyed by viral activity or until the cells had completely degenerated without maintenance fluid change (usually about 14 days).

Hyperimmune serums. Antiserums which had been produced in monkeys, rabbits, guinea pigs, or roosters (for reoviruses) were used. These serums had been prepared for routine use in neutralization tests and had high type-specific neutralization titers. Each serum is diluted 1:5 in 0.85 percent NaCl, and then treated with acid-washed kaolin to remove nonspecific inhibitors. This also diminishes the tendency of HA "shield patterns" to "slip" after formation into the appearance of a false "button." Following kaolin absorption, human erythrocytes are added to remove nonspecific and "cold" hemagglutinins.

Equal volumes of diluted serum and a 25 percent suspension of kaolin in normal saline are mixed, kept for 20 minutes at room temperature, and then centrifuged for 20 minutes at 2,000 rpm. The clear supernate is considered to be a 1:10 dilution of serum. To each milliliter of the latter, 0.1 ml. of a 50 percent suspension of type O cells is added and the mixture kept for 1 hour at 4° C. and again centrifuged for 20 minutes at 2,000 rpm in the cold. If the treated serum is frozen for storage, retreatment with red cells as described above is necessary because cold hemagglutinins may redevelop.

Erythrocytes. Human red blood cells (RBC) of any type may be used for HA tests, but only type O are usable for HAI tests. Little variation between individual donors has been found. To 50 ml. of Alsever's solution, 10–20 ml. of freshly collected blood is added and the suspension centrifuged for 20 minutes at 2,000 rpm. The cells are then washed and recentrifuged three times with dextrose-gelatin-veronal (DGV) solution (*13*) and can be stored for at least 1 week as 10 percent suspension in DGV. For use in HA tests, this RBC suspension is diluted to 0.7 percent, 0.8 percent, 0.9 percent, and 1.0 percent in 0.85 percent NaCl. The smallest number of cells which will produce a definite "button" is desired, and the concentration which will give this result may be determined by trial and error by placing 0.2 ml. of each of these dilutions into tubes and determining the lowest concentration which produces a "button" after 1 hour at 37° C.

Test tubes. Disposable, soft glass Kahn tubes are used in order to avoid spontaneous agglutination or nonspecific inhibition, which may be produced by detergent residues after washing or other chemical contaminants.

Hemagglutination test. All unknown MKCTC isolates are first screened for HA activity. The virus isolate is diluted 1:8 in saline, and 0.4 ml. of this dilution is placed into each of four Kahn tubes, followed by 0.2 ml. of RBC suspension. The tubes are shaken and incubated, two tubes at 4° C. and two at 37° C., for the period of time required for the suspended cells in RBC control tubes to sediment into clear "buttons." This usually takes 1–2 hours. If HA occurs at either or both temperatures, virus titrations are performed at the appropriate temperature. Twofold dilutions are made from 1:8 through 1:1,024. Virus dilutions and RBC suspension are mixed, shaken, and incubated as described above. The end point of titration is the highest virus dilution which causes a definite shield pattern of RBC agglutination at the given temperature, and this dilution is designated as one HA unit. The optimum temperature for maximum HA activity varies among the enterovirus types. These differences are summarized in table 1 from previous reports and our own experience.

Hemagglutination inhibition test. The inhibition test is performed at that temperature which resulted in the highest HA titer for a given isolate. If there was no difference in titer, incubation at 37° C. is preferred because erythrocytes sediment more rapidly at the higher temperature. The HAI test is performed with four HA units per reaction tube. Since only one-half the volume of virus suspension is used in the HAI test as compared with the HA test, that dilution eightfold lower than the HA end point will contain four HA units per 0.2 ml.

For antiserum titration, serial twofold dilutions of the type-specific serum are prepared and 0.2 ml. amounts are placed into Kahn tubes.

To each tube, 0.2 ml. of a known homologous virus suspension containing four HA units is added. The tubes are shaken briefly and incubated for 1 hour at 37° C.; 0.2 ml. of RBC suspension is added, and they are shaken again and incubated at the optimum temperature for the virus type used. Each test must include an antigen titration, and the duration of incubation is determined by observation of the latter. Tests at 37° C. usually require about 45–60 minutes and those at 4° C. about 90–120 minutes. The end point of titration is the highest serum dilution which completely prevents hemagglutination. This dilution is referred to as containing one HAI unit. A satisfactory typing serum should have a titer of 1:160 or greater. Each test should include a serum control to test for the presence of nonspecific hemagglutinins by substituting saline for viral antigen.

For HAI typing of an unknown virus isolate, 0.2 ml. of each typing serum containing four HAI units is added to tubes which have 0.2 ml. of the virus suspension containing four HA units. After shaking and incubation, RBC suspension is added and the tubes are incubated at the appropriate optimum temperature, as previously determined for each isolate. If hemagglutination inhibition is not observed with any serum type, four HA units of the unknown are used in a titration of each serum. If an HAI reaction is still not observed, identification by the neutralization test will be necessary. In this way, new hemagglutinating enterovirus types may be discovered, although it must be remembered that viruses other than enteroviruses which cause hemagglutination with human type 0 cells may be recovered from fecal extracts. Hemagglutinating simian agents may contaminate the tissue culture used for isolation, and mixtures of hemagglutinating and nonhemagglutinating enteroviruses may be isolated from the same fecal specimen.

Table 2. Confirmation by neutralization test of enterovirus and reovirus identifications made by HAI from among field strains

Virus type	Number of strains identified by HAI	Confirming neutralization test	
		Number tested	Number confirmed
ECHO:			
3 (35)	1	1	1
6 (38)	4	4	4
7 (39)	38	17	17
11 (41)	24	14	14
12 (42)	14	14	14
13 (43)	2	2	2
19 (49)	33	15	15
21 (51)	1	1	1
JV–10 (59)	1	1	1
Reovirus:			
1	5	3	3
2	5	2	2
3	6	2	2

NOTE: Revised enterovirus numbers appear in parentheses (reference 1).

Results

During a period of about 1 year, 666 unselected MKCTC enterovirus and reovirus isolates were tested for HA activity as described above. Of these, 134, or about 20 percent, hemagglutinated in sufficient titer to be tested

Table 1. Optimum temperatures for HA and HAI tests performed with strains of enterovirus and reovirus types ordinarily isolated in MKCTC

Virus types with HA titer usually significantly [1] higher at—		Virus types with HA titer usually not significantly different at 4° C. and 37° C.
4° C.	37° C.	
ECHO 3 (35)	Coxsackie B1 (27)	Coxsackie B3 (29)
ECHO 11 (41)	Coxsackie B5 (31)	ECHO 7 (39)
ECHO 13 (43)	ECHO 6 (38)	ECHO 12 (42)
ECHO 19 (49)	Strain JV–10 (59)	Reovirus 1,2,3
ECHO 21 (51)		

[1] Fourfold or greater difference.

NOTE: Revised enterovirus numbers appear in parentheses (reference 1).

by HAI, and all were identified. As shown in table 2, they included all of the recorded hemagglutinating types which are isolated in MKCTC and which readily agglutinate adult human erythrocytes, except Coxsackie B3. Only one "field" strain of Coxsackie B3 which agglutinates adult red cells is known. Coxsackie B1 and B5 strains and other strains of B3 readily agglutinate only fetal erythrocytes, which we did not use.

As also shown in table 2, 76 of the isolates identified by HAI were retested by neutralization typing, and every identification was confirmed. Confirmations included at least one strain of every virus type, and were continued only until we became confident that no false identifications were being made by HAI.

In using the routine technique described, which calls for only a single dilution of typing serum containing four HAI units, we observed no heterologous cross-reactions.

Among the hemagglutinating types encountered in our studies, almost all strains except those identified as Coxsackie B types were HA positive (table 3). Only 11 ECHO strains were HA negative: 4 of ECHO 6, 6 of ECHO 11, and 1 of ECHO 13. Of these, two strains of ECHO 11 and the ECHO 13 strain actually produced some hemagglutination but at too low a titer to permit HAI testing. As mentioned previously, ECHO 6 strains are known to vary in their hemagglutinating capacity, and fetal RBC are necessary for good HA tests with Coxsackie B1, B3, and B5.

Table 3. HA positive and HA negative field strains among known MKCTC hemagglutinating enterovirus and reovirus types

Virus type	Number of strains tested	HA positive	HA negative
Coxsackie:			
B1 (27)	2	0	2
B3 (29)	44	0	44
B5 (31)	34	0	34
ECHO:			
3 (35)	1	1	0
6 (38)	8	4	4
7 (39)	38	38	0
11 (41)	30	24	6
12 (42)	14	14	0
13 (43)	3	2	1
19 (49)	33	33	0
21 (51)	1	1	0
JV–10 (59)	1	1	0
Reovirus:			
1	5	5	0
2	5	5	0
3	6	6	0

NOTE: Revised enterovirus numbers appear in parentheses (reference 1).

Table 4. Failure of hemagglutination among MKCTC field strains belonging to enterovirus types not presently known to hemagglutinate

Virus type	Number of strains tested (all found negative)	Virus type	Number of strains tested (all found negative)
Poliovirus:		ECHO—Con.	
1 (1)	14	5 (37)	10
2 (2)	7	9 (40)	12
3 (3)	40	14 (44)	26
Coxsackie:		15 (45)	21
A9 (12)	44	16 (46)	4
A16 (19)	6	17 (47)	26
B2 (28)	24	18 (48)	3
B4 (30)	65	20 (50)	17
B6 (32)	6	22 (52)	5
ECHO:		23 (53)	1
1, 8 (33)	57	24 (54)	1
2 (34)	7	27 (57)	21
4 (36)	24		

NOTE: Revised enterovirus numbers appear in parentheses (reference 1).

During the period covered by this study, we performed HA tests on 441 MKCTC isolates of types not presently known to produce hemagglutination. These are summarized in table 4. No hemagglutinating strains were found.

Discussion

As a result of our experience with the routine use of HA and HAI tests for enterovirus and reovirus identification, we believe that this method of virus typing is highly useful, and we must disagree with a recent report from another laboratory (*14*) which contained the opinion that hemagglutination as a screening procedure contributes little to the identification of ECHO virus strains.

The tests are simple to perform and results are rapidly obtained. A large number of isolates can be screened by one technician in a morning, and the HA positive strains can be identified by him during the same day. This results in much more prompt answers at much less expense than is possible with neutralization

typing techniques. The tests are readily adapted to the use of the microtiter apparatus (*15*), resulting in a marked saving of expensive typing antiserums. There is even some sparing of time and expense with the HA negative strains. In typing the latter by neutralization, we eliminate two antiserum pools, which consist of the hemagglutinating ECHO types, in the preliminary test. About 80 percent of HA negative strains are identified by the remaining serum pools. The untyped 20 percent consists of strains for which antiserums are not included in pools, those which will be found untypable, and the few HA negative strains of hemagglutinating types. Because the latter do occur, it is necessary to check these strains by the neutralization test with the appropriate antiserums before recording them as untypable.

Reoviruses are difficult to identify by the neutralization test even as ECHO 10, because they are so slow in producing a cytopathic effect in MKCTC and because the infectious titer of an isolate is often much higher than the apparent titer indicated by cytopathic effect. HA and HAI tests are a practical necessity if specific-type designation is desired.

Occasionally, typing by the hemagglutination method may be of unusual value. After the end of the oral poliovirus vaccination campaign in Atlanta, Ga., in 1961, ECHO 19 became the predominant enterovirus, and ECHO 7 was frequent, together with other nonhemagglutinating types. During the last 6 months of the year, 40 percent of the viruses isolated in our studies of that vaccine program (*16*) were identified by HAI tests. In the study of a large epidemic produced by a hemagglutinating strain, a great saving in cost may result from the use of this method, and the facts of the investigation may be elucidated much more quickly.

Summary

In the Enterovirus Unit of the Communicable Disease Center, simple, routine methods of hemagglutination and hemagglutination inhibition testing of enteric virus isolates resulted in the identification of 20 percent of 666 unselected isolates during a 1-year period and 40 percent of isolates during a period of ECHO 19 predominance in Atlanta, Ga. No heterologous cross-reactions were noted, and no false identifications were found among those isolates retested with the neutralization test. Because the method described saves time and expense, it is recommended as a first step in the routine typing of enteroviruses and reoviruses.

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Congress of Dermatologists

A simple skin test to determine sensitivity to penicillin is being developed and evaluated as a safeguard against anaphylaxis. Fairly accurate results in tests with penicilloyl-polylysine were reported to the 12th International Congress of Dermatologists held in Washington, D.C., in September 1962.

The reagent, developed by a group of workers at the Washington University School of Medicine, was tested initially on 1,250 subjects, mainly patients of the St. Louis Venereal Disease Clinic.

Of 59 patients with a history of allergic reactions to penicillin, 76 percent (45) reacted positively to penicilloyl-polylysine; about 4 percent (44) of the 1,191 with no allergic history were positive. None of the 1,147 patients with no allergic history and negative reactions to the test reacted to subsequent penicillin treatment. (These results were reported by Dr. Charles W. Parker, Dr. Jack Shapiro, Dr. Milton Kern, and Dr. Herman Eisen in the April 1962 issue of the *Journal of Experimental Medicine*, pp. 821–838.)

Further investigations in eight venereal disease clinics showed less clear-cut results than the initial studies. Dr. W. G. Simpson, chief of epidemiology and immunization, Division of Foreign Quarantine, Public Health Service, who is coordinating the studies in cooperation with the group at the Washington University School of Medicine, presented the results.

He reported that the percentage of positive reactions to the test substance in 330 patients with a history of sensitivity to penicillin was 36 percent. One percent of the 2,542 persons with a negative skin test reacted allergically when given penicillin.

Dr. Simpson told the conferees that he considered test results significant enough "to warrant optimism on the eventual usefulness of the test."

Dr. Harold F. Blum, a National Cancer Institute biologist, discussed the bearing on skin cancer in man of experiments on cancer induced in mouse skin by ultraviolet light. These experiments support other evidence that sunlight is a major factor in human skin cancer. Dr. Blum pointed out, however, that there is no need to "shun sunlight entirely, but only to avoid the tiny fraction that ordinarily affects the skin," a fraction removed by window glass and virtually lacking from early morning and late afternoon sunlight, even in midsummer.

"The dangerous thing," Dr. Blum told dermatologists, "is continued exposure such as occurs in outdoor workers." He reported that the development of skin cancer, at least that induced by ultraviolet light, is cumulative, beginning with the first dose or shortly thereafter. He concludes that in the population as a whole only a certain proportion may be expected to develop cutaneous cancers within the normal span of life—depending upon the amount of exposure to sunlight, individual susceptibility, and luck.

Physicians from 17 countries reported to the congress on skin disease in their own areas. Many of the cases presented were unique. On three afternoons of the 5-day congress, case reports were presented in a series of 34 motion pictures in sound and color, each compiled from physicians' original photography. According to Dr. Marion B. Sulzberger, Washington, D.C., chairman of the International Committee of Dermatology, this was "the first time interesting and characteristic cases from all over the world had been made available in this form to an international gathering of physicians."

The 2,200 registrants at the congress came from 51 countries, including most European and Latin American nations, Japan, Burma, Egypt, Iran, Singapore, Turkey, the Republic of Senegal, the U.S.S.R., and Poland. The Secretary-General of the congress, Dr. Clarence S. Livingood, Detroit, Mich., credited the jet age for the record attendance.

Official languages were English, French, German, and Spanish. Three teams of translators interpreted most of the scientific presentations from the languages in which they were presented into the other three tongues.