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Standardized Viral Hemagglutination and Hemagglutination-Inhibition Tests

II. Description and Statistical Evaluation

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Standardized hemagglutination and hemagglutination-inhibition procedures are described and statistically evaluated for all animal viruses where applicable, except for rubella and the arbovirus group. The standardized tests employ a constant phosphate-buffered saline diluent and constant volumes of serum, antigen, and standardized erythrocyte suspension. The standardized hemagglutination test has a reproducibility of 84 to 96% with adenoviruses, rubeola, and the myxoviruses, and 78 to 93% with reoviruses; the standardized hemagglutination-inhibition test has a reproducibility of 95 to 100% with all viruses tested.

The standardized hemagglutination (HA) and hemagglutination-inhibition (HI) tests consist of two distinct operations: (i) the preparation of standardized erythroctye suspensions, which was described in the preceding paper (5); and (ii) the performance and evaluation of the tests, presented in this report. Current HA-HI procedures include a great variety of diluents, volumes, and serum treatments, many of which can be reduced to constant methods. The primary goals of standardizing viral HA-HI tests are therefore to simplify the tests and to provide a statistically sound procedure which is applicable to as many virus-cell-serum systems as possible.

Salk's original HA-HI tests (19) have been applied to many other virus-cell or virus-serum-cell systems since the discovery of viral hemagglutination by Hirst in 1941. A partial list includes the HA-HI tests for adenoviruses (17, 18), enteroviruses (4, 10, 11), reoviruses (3, 14), myxoviruses (9, 13, 15), poxviruses (8, 26), and some of the murine viruses (12, 24). The reproducibility of any of these HA or HI methods, considered as a test procedure, has not been reported in depth. Evaluations of HA-HI tests have been reported, however, by way of comparison of the diagnostic results of the HA-HI tests to serum neutralization and complement-fixation tests (4, 16, 20, 26).

The standardized HA and HI tests described in this report will apply to the following viruses: the hemagglutinating strains of coxsackie and echoviruses; the murine encephalomyelitis viruses; reovirus types 1, 2, and 3; influenza A, B, and C; mumps; Newcastle disease virus (NDV); all strains of parainfluenza 1, 2, 3, and 4; murine pneumonia virus (PVM); two strains of coronavirus; rubeola; smallpox, vaccinia, and other variola-like viruses; ectromelia of mice; adenovirus groups I, II, and III; polyoma and K virus; the murine H viruses, X14, and rat virus (RV). The standardized tests can also be used for other lower animal viruses and for some of the Bedsonia group which hemagglutinate red blood cells. We have not included rubella and the arboviruses in the standardization efforts at this time but hope to do so at a later date.

Additional information on the hemagglutination properties of the viruses discussed in this report may be found in several excellent reference works (1, 7, 13, 25). These works should be consulted for details on virus strain peculiarities and preparation of HA antigens, and for the complete lists of references included with the pertinent chapters.

MATERIALS AND METHODS

Microtiter technique. The standardized HA-HI tests were performed in the microtiter system described by Sever (21). Flexible vinyl "U" and "V" plates (Cooke Engineering Co., Alexandria, Va.; no. 220-24 and 220-25, respectively) and rigid styrene "U" and "V" plates (Linbro Chemical Co., New Haven, Conn.; no. IS-MRC-96 and IS-MVC-96, respectively) were evaluated during the development of the tests. Antigens and sera were diluted in the wells with stainless steel "tulip" loops (Cooke no. 220-33 and 220-34). At the start of the incubation period, the plates were sealed with transparent tape (Cooke no. 220-12) to retard evaporation.

Diluents. A large number of diluents were evaluated (*see* Table 1) to find the one best suited or at

least adequate for all systems studied in this report. The one ultimately chosen was 0.01 M phosphate-buffered saline (PBS), *p*H 7.2, containing 0.010 M total PO_4 and 0.146 M NaCl, and with an ionic strength of 0.171. This buffer was modified by the addition of adenovirus type 6 equine antiserum (adsorbed with rat erythrocytes) to a final concentration of 1.0% by volume for HA-HI tests with the group III adenoviruses, or by the addition of bovine serum albumin (BSA) fraction V powder to a final concentration of 0.1% (w/v) for HA-HI tests with several of the murine viruses. The basic PBS diluent was used throughout the study for preparing red blood cell (RBC) suspensions, virus dilutions in the HA test, serum dilutions, and the 8 HA unit virus suspension in the HI test; it also served as the diluent in the kaolin and receptor-destroying enzyme (RDE) treatments of sera.

Antigens and antisera. The antigens used throughout this study were harvests of virus-infected tissue culture or embryonated chicken eggs. All harvest materials were clarified by centrifugation at 1,200 × *g* for 10 min and at 4 C. The viruses used were prototype strains procured from the Biological Reagents Section, Laboratory Division, National Communicable Disease Center (NCDC).

Antisera used in the HI test were procured from the Biological Reagents Section, NCDC. All sera were heated at 56 C for 30 min and initially diluted 1:5 or 1:10 in PBS. Where applicable, the sera were further treated by adsorption with the erythrocytes to be used in the test, by kaolin extraction, or by the RDE of *Vibrio cholerae*. RBC adsorption was carried out at 4 C for 1 hr with 0.1 ml of the appropriate 50% RBC suspension per ml of starting serum dilution. The cells were resuspended frequently and finally removed by centrifugation. For kaolin extraction, equal volumes of 25% acid-washed kaolin in PBS and 1:5 serum were mixed and incubated at room temperature for 20 min. The serum was recovered after centrifugation and was considered to be a 1:10 dilution. For RDE treatment, one volume of undiluted serum was mixed with four volumes of RDE (100 units/ml) and incubated overnight in a water bath at 37 C. Three volumes of 2.5% trisodium citrate (dihydrate) was added. The mixture was heated at 56 C for 30 min, and the serum was raised to a starting dilution of 1:10 by the addition of two volumes of PBS. These treatments were sufficient to remove or inactivate the nonspecific inhibitors and natural hemagglutinins from all of the sera used in this study, regardless of source.

RBC suspensions. Sheep, guinea pig, human "O", rhesus and vervet monkey, rat, mouse, and chicken bloods were collected in Alsever solution (5). The red cells were washed in PBS and adjusted to 0.40% suspensions for mammalian cells and 0.50% for chicken cells by the spectrophotometric standardization procedure (5).

RESULTS

Selection of buffered diluent. In an effort to find a single diluent which would be compatible with all hemagglutinating systems, we evaluated 29 buffered diluents by microtiter HA with viruses representing the different groups. The diluents investigated are listed in Table 1. The viruses tested were adenoviruses 5, 7a, and 9, influenza A and B, parainfluenza 1 and 3, mumps, rubeola, echovirus 6, reoviruses 2 and 3, and vaccinia. The erythrocytes of each species used in the evaluation (rat, rhesus monkey, guinea pig, human "O," and chicken) were suspended in each of the buffers so that both components of each HA test would be diluted in the same buffered diluent.

For each virus, the diluents were compared with one another by HA titer, by ease of reading the end point, and by the appearance of the cell controls. Observations on hemolysis, nonspecific agglutination, poor settling, and other factors were carefully recorded. In addition, sufficient data were obtained to afford evaluation of the type of microtiter plate best suited for the different species of erythrocytes used in the test.

The data are too extensive to be tabulated here; hence, only descriptive results will be given. Gelatin at 0.1 to 1.0% concentrations could not be substituted for BSA because it gave false agglutination patterns in the cell controls. Diluents containing BSA or very low concentrations of gelatin (0.005 to 0.01%) were suitable, although unnecessary, for adenovirus, enterovirus, and reovirus HA tests, but were not suitable for myxovirus HA tests with chicken cells. The protein additives in general hastened the settling of the cells, but did not enhance the hemagglutination titer and in several systems exhibited ragged, fuzzy, and erratic cell controls. Such patterns made the end point of a virus titration difficult to read.

The barbital, EDTA, HEPES-saline, TES, and TRIS buffers at all *p*H values gave low titers for adenoviruses tested with rat cells. Lower titers were also observed at *p*H 8.8 than at the other *p*H levels in most of the virus-cell systems, especially adenovirus (rat), echovirus (human "O"), and influenza A (chicken).

Several of the buffers proved to be unsuitable media for storage of the RBC suspensions. Rat, monkey, guinea pig, human "O," and chicken erythrocytes began to hemolyze after 1 day at 4 C in the following buffers: barbital, *p*H 8.8; dextrose-gelatin-Veronal, *p*H 7.4; HEPES, *p*H 8.0; TES, *p*H 8.0; and TRIS, *p*H 8.0 and 8.8.

No difference in titer was noted in any virus-cell system among the phosphate buffers, dextrose-gelatin-Veronal buffer, or HEPES-gelatin buffer, or at any *p*H between 6.2 and 8.0. The selection of a buffered diluent to use throughout the HA-HI procedure was therefore made from this group, and was based on considerations of simplicity in preparation, storage at room temperature, sta-

TABLE 1. *Composition of the diluents evaluated*

No.	Name	Composition
1	Bovine albumin-borate saline	0.042 M boric acid-NaOH buffer, 0.10 M NaCl, 0.4% BSA; *p*H 9.0
2–5	Barbital (Veronal)	0.01 M sodium barbital, 0.14 M NaCl; adjusted to *p*H 6.2, 7.2, 8.0, and 8.8 with 0.2 M HCl
6	Dextrose-gelatin-Veronal	0.00315 M barbital, 0.00184 M sodium barbital, 0.06% gelatin, 0.00018 M $CaCl_2$ anhydrous, 0.000486 M $MgSO_4 \cdot 7H_2O$, 0.145 M NaCl, 0.056 M glucose; *p*H 7.4
7	EDTA (Versenate)	0.01 M sodium ethylenediaminetetraacetate, 0.14 M NaCl; adjusted to *p*H 7.2 with 1 N NaOH
8–10	HEPES-saline	0.01 M HEPES (N-2-hydroxyethylpiperazine-*N*-2-ethanesulfonic acid), 0.14 M NaCl; adjusted to *p*H 6.2, 7.2, and 8.0 with 1 N NaOH
11	HEPES-gelatin	0.025 M HEPES, 0.15 M NaCl, 0.001 M $CaCl_2 \cdot 2H_2O$, 0.005% gelatin; adjusted to *p*H 6.2 with 1 N NaOH (N. J. Schmidt, California State Department of Public Health, Berkeley, *personal communication*)
12	Phosphate-buffered saline	0.005 M phosphates ($NaH_2PO_4 \cdot H_2O$ + Na_2HPO_4 anhydrous), 0.146 M NaCl; *p*H 7.2
13–16	Phosphate-buffered saline	0.01 M phosphates (as in 12) in various proportions to achieve *p*H 6.2, 7.2, 8.0, and 8.8; 0.14 M NaCl
17	Phosphate-buffered saline	0.038 M phosphates (as in 12), 0.109 M NaCl; *p*H 7.2
18	PBS with protein additives	Same as 17, containing adenovirus 6 rat RBC-adsorbed antiserum to 1.0% final concentration
19	PBS with protein additives	Same as 17, containing 0.1% BSA
20–22	PBS with protein additives	Same as 17, containing 1.0, 0.5, or 0.1% gelatin
23	Bacto-hemagglutination buffer	Sodium chloride, disodium phosphate, and potassium dihydrogen phosphate; *p*H 7.3 (code 0512, Difco Laboratories)
24–26	TES	0.01 M TES [*N*-tris(hydroxymethyl)methyl-2-aminomethanesulfonic acid], 0.14 M NaCl; adjusted to *p*H 6.2, 7.2, and 8.0 with 1 N NaOH
27–29	TRIS	0.01 M tris(hydroxymethyl)aminomethane, 0.14 M NaCl; adjusted to *p*H 7.2, 8.0, and 8.8 with 0.2 M HCl

bility, suitability for erythrocyte suspensions, and use in serum treatments. We chose phosphate-buffered saline containing 0.01 M total phosphates (0.00772 M Na_2HPO_4 anhydrous + 0.00228 M $NaH_2PO_4 \cdot H_2O$) and 0.146 M NaCl at *p*H 7.2 as the diluent which best fits these criteria.

The four types of disposable microtiter plates listed in Materials and Methods were evaluated in the various buffer-virus-cell systems. The flexible "U" plates were unsuitable for adenovirus HA tests with rat cells, because the cells often failed to settle evenly. These plates did appear to be suitable for HA tests with the other mammalian cells. However, the styrene "U" plates were adequate for all mammalian cell HA tests and, in addition, were easier to read than the vinyl plates. "V" plates could be employed for mammalian and avian red cells but usually gave a lower end point owing to slippage of the cells into the bottom of the "V."

Ratio of cell to antigen volumes in the HA test. Most of the present variations in concentration of RBC suspensions, volume of the RBC suspension, and volume of antigen can be eliminated without sacrifice of accuracy or convenience. There is no difference in total cell number between 0.025 ml of 1.0% cells and 0.05 ml of 0.5% cells. The use of 0.05 ml of a 0.4% RBC suspension, however, results in 20% fewer cells in each well. The lower concentration is nonetheless preferred over a 0.5% suspension for mammalian cells because it enhances sensitivity at the end point and partially corrects for the size difference between avian and mammalian cells. Avian cells have an approximately 40% greater "mean corpuscular volume" than mammalian cells; hence,

0.05 ml of a 0.5% avian cell suspension contains about the same number of cells as 0.05 ml of a 0.4% mammalian cell suspension (2×10^6 to 5×10^6 cells/0.05 ml).

Comparative tests were conducted to determine whether 0.05 ml of a 0.4% mammalian cell suspension could substitute for 0.025 ml of a 1.0% suspension of cells. Adenoviruses 3, 5, 7, 9, 10, and 13, echovirus 6, and reovirus 3 were titrated in both systems in duplicate. In every test, the end point of agglutination in the 0.4% system was clearly readable and was the same or one dilution higher than the titer in the 1.0% system. We consistently noticed a higher titer for the adenoviruses when 0.05 ml of 0.4% suspensions was employed. However, the larger volume required an additional 5 min for the cells to settle. It therefore appears that the 0.4% cell system is more sensitive than the 1.0% system for mammalian cells.

On the basis of these results, the standardized HA test was designed to employ avian cells at a 0.5% concentration and mammalian cells at 0.4%. The microtiter HA system would then employ 0.05 ml of cells added to 0.05 ml of virus dilution. The microtiter HI test would employ 0.025 ml of serum dilution plus 0.025 ml of the virus suspension containing 4 HA units (8 HA units per 0.05 ml); after 40- to 60-min incubation period, 0.05 ml of the appropriate cell suspension would be added.

Treatment of sera for the HI test. Longstanding procedures for adenovirus and measles (rubeola) HI tests include the extraction of sera with kaolin (13, 18). Preliminary observations in our laboratory, however, indicated that kaolin treatment might not be necessary as a routine procedure. We conducted a series of experiments to establish this point.

Ninety-five preimmune horse sera from an adenovirus 1 to 31 immunization series were tested by HI against 28 of these adenovirus types. The sera were heated at 56 C for 30 min, diluted 1:5 as the starting dilution, and adsorbed with 50% rat or rhesus erythrocytes. Kaolin was not used. In 2,700 tests, none of these preimmune sera showed any nonspecific inhibition activity.

Further testing was done with human, rabbit, and guinea pig preimmune sera. Several hundred human sera, 60 rabbit sera, and 40 guinea pig sera have been tested over a 3-year period with many adenovirus types. Again, these were heat-treated and adsorbed with the appropriate red cells but were not adsorbed with kaolin. In approximately 2,000 tests, none of these preimmune sera contained nonspecific viral HA inhibitors that were not removed by heating and cell adsorption.

Similar experience has suggested that kaolin treatment is not required for measles HI tests. During the same 3-year period, over 700 human, 25 monkey, and 10 goat sera have been used satisfactorily in measles HI tests with heating and cell adsorption but without kaolin.

The use of kaolin or similar treatment, such as Rivanol (23), is necessary for enterovirus and reovirus human and animal sera, with the possible exception of reovirus chicken antiserum, which appears to be best treated by human RBC adsorption instead of kaolin or kaolin plus erythrocytes. RDE generally replaces the heat-trypsin-periodate treatment formerly employed for myxovirus HI tests (15).

Adsorption of the serum with the species of red cells to be used in the HI test is often necessary. Three general rules may be followed. (i) If rhesus, vervet, rat, mouse, or guinea pig red cells are used, the sera from any source must be adsorbed to remove natural agglutinins. (ii) If human, chicken, or sheep cells are used, the sera need not be adsorbed on a routine basis, but should be so treated if the serum controls show nonspecific agglutination. (iii) In HI tests employing red cells from the same species as the serum being tested, adsorption is generally not required. Cases in point are diagnostic enterovirus, reovirus, influenza, and parainfluenza HI tests in which human sera are tested with human "O" cells; influenza strain analyses in which chicken hyperimmune sera are tested with chicken cells; parainfluenza cross-HI tests in which guinea pig immune sera are tested with guinea pig cells; and PVM HI tests in which mouse sera are tested with pooled mouse erythrocytes.

Standardized HA-HI tests. On the basis of the foregoing results, the standardized HA-HI tests were established as follows. The microtechnique may be performed in any microtiter plate which the user finds suitable. Although no single type of plate presently available is *ideal* for all virus-cell combinations, we have found the rigid styrene "U" plate to be adequate for all of the viruses and red cells discussed in this report. Therefore, at present we prefer this plate for the standardized HA-HI tests.

The diluent used throughout these test procedures is 0.01 M PBS, *p*H 7.2, except for two modifications: PBS/HS (PBS with 1.0% heterotypic serum—usually adenovirus 6 antiserum) for the group III adenoviruses, and PBS/BSA (PBS with 0.1% bovine serum albumin, fraction V) for

TABLE 2. *Outline of the necessary variables in the standardized HA-HI tests*

Virus	Erythrocyte			Serum treatment for HI
	Species	Concn	Incubation temp	
Enteroviruses[a]				
Coxsackie A-20, A-21, A-24; echovirus 3, 11, 13, 19; mouse encephalomyelitis	Human "O"[b]	0.4	4	56 C, 30 min, kaolin adsorption
Coxsackie B-1, B-3, B-5; echovirus 6, 7, 12, 20, 21, 24, 29, 30, 33	Human "O"[b]	0.4	37	56 C, 30 min, kaolin adsorption
Coxsackie A-7	Chicken[b]	0.5	24	56 C, 30 min, kaolin adsorption
Reovirus 1, 2, 3	Human "O"[b]	0.4	24	56 C, 30 min, kaolin adsorption
Influenza A and B (fresh isolates); NDV, parainfluenza 1, 2, 3; Sendai; SV-5, SV-41, SF-4; mumps	Guinea pig,[c] human "O"	0.4	24	RDE-heat; RBC adsorption for guinea pig cells
Parainfluenza 4A and 4B[d]	Rhesus, guinea pig	0.4	24	RDE-heat, RBC adsorption
Influenza A and B (laboratory-adapted); NDV; mumps; many parainfluenzas	Chicken	0.5	24	RDE-heat
Influenza C	Chicken	0.5	4	RDE-heat
PVM	Mouse	0.4	24	56 C, 30 min
Coronavirus (OC 38 and OC 43)[e]	Chicken	0.5	24	56 C, 30 min
	Rat[b] or mouse	0.4	24	56 C, 30 min, RBC adsorption
Rubeola (measles)	Vervet, rhesus[b]	0.4	37	56 C, 30 min, RBC adsorption
Smallpox, vaccinia, ectromelia	Chicken[b]	0.5	24	56 C, 30 min
Adenovirus, human and simian				
Group I	Rhesus	0.4	37	56C, 30 min, RBC adsorption
Groups II, III	Rat[b, c]	0.4	37	56 C, 30 min, RBC adsorption
Polyoma	Guinea pig	0.4	4	RDE-heat
K virus	Sheep	0.4	4	56 C, 30 min
Rat virus, X14, the H viruses	Guinea pig	0.4	24	56 C, 30 min, kaolin or RBC adsorption

[a] The hemagglutinating enteroviruses may require PBS at *p*H 5.8, 6.9, or 7.4 for optimal HA titers (10). In addition, many wild strains of certain of these enteroviruses do not hemagglutinate at any *p*H or temperature.

[b] Animals must be selected for erythrocytes sensitive to viral hemagglutination.

[c] Red cells should be used within 2 days after collection.

[d] Parainfluenza 4A and 4B HA antigens are prepared by an ether-Tween 80 procedure (G. E. Killgore and W. R. Dowdle, *manuscript in preparation*).

[e] Mouse brain passages of both strains of the human "IBV-like" virus (a coronavirus) have recently been shown to possess HA antigens of diagnostic value (H. S. Kaye and W. R. Dowdle, *manuscript in preparation*).

GD VII, polyoma, and PVM. This diluent may be used for other viruses if necessary.

Cell suspensions are standardized spectrophotometrically to a final concentration of either 0.40% (containing 4×10^7 to 9×10^7 cells/ml) for mammalian red cells or 0.50% (containing 3×10^7 to 8×10^7 cells/ml) for avian red cells.

Standardized HA test (microtiter): Use 0.05 ml of virus dilution in a 1:5, 10, 20 ··· series plus 0.05 ml of RBC suspension per well; incubate for 1 to 2 hr at the prescribed temperature (Table 2) and read for end point of hemagglutination.

Standardized HI test (microtiter): Treat serum as outlined (Table 2) or with further adsorptions if necessary; use 0.025 ml of serum dilution in a 1:10, 20, 40 ··· series plus 0.025 ml of virus dilution containing 4 HA units per well. Incubate for 40 to 60 min at room temperature; add 0.05 ml

of RBC suspension per well, and reincubate for 1 to 2 hr at the prescribed temperature. Read for end point of inhibition of hemagglutination.

Macrotiter or tube tests are performed in 13 × 100 mm Wassermann tubes and contain 10 times the microtiter volumes.

STATISTICAL EVALUATION OF STANDARDIZED HA-HI TESTS

Reproducibility. In most tests in which a twofold dilution scheme is used, fourfold or greater changes in titer (differences of two or more dilutions) on serial specimens from one patient (e.g., acute and convalescent sera) are considered diagnostically or clinically significant. That is, changes in titer greater than twofold (one dilution) are implicitly and explicitly interpreted as "real." Therefore, a useful expression for reproducibility of titers should be related to this criterion of clinical significance.

This can be done by determining on an appropriate number of blind duplicate specimens the percentage of duplicate titers that differ by two or more dilutions (fourfold or greater changes). This percentage is the false-positive rate for the test based on fourfold or greater changes. The reproducibility of the test can be expressed as the converse of the false-positive rate—that is, the percentage of duplicate titers that are the same (excluding specimens negative on both duplicates) or differ by one dilution. Duplicates analyzed on the same day (run) can be used to estimate within-day reproducibility; duplicates analyzed on different days can be used to estimate day-to-day reproducibility. This is the approach used in this evaluation.

Within-day and day-to-day reproducibility of the standardized HA and HI microtiter tests were evaluated by use of coded "blind" specimens.

Experimental design of adenovirus-measles and myxovirus tests. Estimates of reproducibility for the adenovirus, rubeola, and myxovirus tests were obtained from a minimum of 100 duplicate determinations (a total of >200 tests) of both virus and serum titers. The HA tests were performed on duplicate samples of 14 or 15 positive and 3 negative antigens on each of the first 8 days; the HI tests were performed on duplicate samples of 12 positive and 2 negative sera on each of the second 8 days. HA and HI determinations were both made for 8 days each; two different lots of basic PBS buffer and four lots of red cells were used (Fig. 1). Buffer-lot 1 was used for the first 4 days with a different lot of red cells each day (four animals). Buffer-lot 2 was used for the following 4 days with fresh lots of cells from the same four animals. This pattern was repeated for buffer lots 3 and 4 in the HI test. Altogether, eight lots of cells and four lots of buffer were included in the evaluation. Thus, we obtained information on variables that may affect the routine reproducibility of titers.

Normal laboratory conditions were further simulated by using antigens and antisera from various sources covering a wide range of titers. Tissue culture harvests of adenovirus types 4, 5, 6, 8, 9, 10, 13, 17, 19, 22, and 24 were titrated with rat red cells; types 7a, 20, 21, and measles, with rhesus monkey red cells; and three negative controls (adenovirus types 12 and 18 and a HEp-II tissue control), with either rat or rhesus erythrocytes. The 15 positive antigens covered HA titers from 1:20 to 1:5,120; the three negative controls were consistently <1:2.5. In the HI test, two lots each of adenovirus equine antiserum types 4, 5, 7a, 9, and 17 and three measles antisera (goat, monkey, and human convalescent plasma) were titrated with the homologous virus and the appropriate rat or rhesus red cells. Two human acute-phase sera were used as negative controls. The 12 positive sera represented HI titers from 1:80 to 1:2,560, and the two negative controls were <1:10.

The myxovirus HA tests were performed on two or three lots each of tissue culture harvests of parainfluenza type 1 (C-35), type 1 (Sendai), type 2 (Greer), type 3, mumps, and influenza B (B/Georgia/1/61 and B/Georgia/14/62), and on allantoic fluid harvests of influenza A_2/Japan/305/57. These viruses represented a total of 14 antigens with a titer range of 1:10 to 1:1,280. Three monkey kidney and HEp-II tissue antigen controls (<1:5) were also included. The HI tests were performed on three lots each of equine antisera against parainfluenza types 1, 3, and mumps; on two lots each of influenza A and B chicken antisera; and on two human acute-phase sera tested with influenza A and B for negative

	HA test								HI test							
	Buffer lot 1				Buffer lot 2				Buffer lot 3				Buffer lot 4			
Cell:	C_1	C_2	C_3	C_4	C_1	C_2	C_3	C_4	C_5	C_6	C_7	C_8	C_5	C_6	C_7	C_8
Day:	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16

FIG. 1. *Experimental design for evaluation of standardized HA-HI tests with adenovirus, rubeola, and myxovirus.*

controls. The titer range represented by the 12 positive sera was 1:80 to 1:1,280, and the two negative sera were consistently <1:10. All myxovirus HA and HI tests were performed with guinea pig cells.

Results of adenovirus-measles and myxovirus tests. The agreement of duplicate titers obtained on the same day for each test (HA and HI) and virus group is summarized in Table 3. Based on fourfold or greater differences, the false-positive rate for each of these tests was less than 5%. Hence, reproducibility of titers for specimens processed on the same day is greater than 95%. The percentage of duplicates with the *same* titer differed significantly (chi-square test with P = 0.05) for each test and virus group; duplicate HI titers were more frequently identical than were duplicate HA titers. Within each virus group, within-day agreement of the HI test was significantly higher than that of the HA test. In addition, myxovirus titers for both HA and HI tests were more reproducible than adenovirus-measles titers.

The agreement of duplicate titers obtained on different days with the use of different cells each day is summarized in Table 4. Other components (for example, buffer) of the test remained unchanged. As expected, the effects of day-to-day variation and different cell lots decreased the reproducibility of titers. In both tests and virus groups, the percentage of duplicates with the same titer was lower for day-to-day duplicates than for within-day duplicates. Day-to-day reproducibility was lower than within-day reproducibility by as much as 10.6 percentage points for the HA tests on the adenoviruses and rubeola; however, this relatively large decrease was due primarily to one lot of cells that yielded titers consistently higher than the other three lots. As with the within-day results, day-to-day agreement of the HI test was better than that of the HA test, and myxovirus titers were more reproducible than adenovirus-rubeola titers.

The results in Table 5 are similar to those in Table 4 except that agreement of duplicate titers was affected by different lots of the buffer rather than different cells on each day. The use of different buffer lots affected day-to-day reproducibility about as much as did the use of different cells. With the exception of adenovirus and measles HA titers, the percentages in similar categories in Tables 4 and 5 are equal. Again, the HI test and the myxovirus HA and HI titers appear to be more reproducible than the HA test and the adenovirus or rubeola HA and HI titers.

Experimental design of reovirus tests. Estimates of reproducibility were obtained from approximately 60 duplicate determinations of both hemagglutinin and serum titers. The HA tests were performed on duplicate samples of 10 positive and 2 negative specimens with each of two cell lots on each of 3 days; the HI tests were performed on duplicate samples of 14 positive and 2 negative sera with each of two cell lots on each of 2 days. One lot of basic PBS buffer and two lots of cells were used altogether, as indicated in Fig. 2. The two lots of human "O" erythrocytes were collected at the start of the evaluation and were used throughout the week of testing, with a portion being washed and standardized each day.

The antigens consisted of four lots of reovirus type 1, four of type 2, and two of type 3, all harvested from rhesus or A rican green monkey kidney tissue culture. The HA titer range was 1:10 to 1:160 for the positive antigens and was consistently <1:2.5 for two negative control anti-

TABLE 3. *Within-day reproducibility of adenovirus, measles, and myxovirus tests*

Agreement[a] of duplicate titers obtained on same day	Adenovirus-rubeola				Myxovirus			
	HA		HI		HA		HI	
	No. of pairs	Per cent	No. of pairs	Per cent	No. of pairs	Per cent	No. of pairs	Per cent
Same titer	53	43.8	65	67.7	65	58.0	87	90.6
One dilution different	62	51.2	28	29.2	43	38.4	9	9.4
Two dilutions different	5	4.2	3	3.1	4	3.6	0	—
Three dilutions different	1	0.8	0	—	0	—	0	—
Total no. of duplicates	121	100.0	96	100.0	112	100.0	96	100.0
Reproducibility[b]	95.0%		96.9%		96.4%		100.0%	

[a] Antigens and sera whose duplicate titers were negative are excluded to avoid artificially over-estimating the reproducibility.

[b] Reproducibility is defined as the percentage of duplicate titers that are the same or differ by one dilution in either direction (excluding negative pairs).

TABLE 4. *Day-to-day reproducibility of adenovirus, measles, and myxovirus tests (agreement of titers on different days using different cells)*

Agreement[a] of duplicate titers obtained on different days; $cell_1$ vs. $cell_{2,3,4}$ or $cell_5$ vs. $cell_{6,7,8}$	Adenovirus-rubeola				Myxovirus			
	HA		HI		HA		HI	
	No. of pairs	Per cent	No. of pairs	Per cent	No. of pairs	Per cent	No. of pairs	Per cent
Same titer	68	38.0	88	61.1	83	49.4	131	78.0
One dilution different	83	46.4	50	34.7	70	41.7	37	22.0
Two dilutions different	25	14.0	6	4.2	12	7.1	0	—
Three dilutions different	3	1.7	0	—	3	1.8	0	—
Total no. of duplicates	179	100.1	144	100.0	168	100.0	168	100.0
Reproducibility[b]	84.4%		95.8%		91.1%		100.0%	

[a] See Table 2, footnote *a*.
[b] See Table 2, footnote *b*.

TABLE 5. *Day-to-day reproducibility of adenovirus, measles, and myxovirus tests (agreement of titers on different days using different lots of PBS buffer)*

Agreement[a] of duplicate titers obtained on different days; $buffer_{1,3}$ vs. $buffer_{2,4}$	Adenovirus-rubeola				Myxovirus			
	HA		HI		HA		HI	
	No. of pairs	Per cent	No. of pairs	Per cent	No. of pairs	Per cent	No. of pairs	Per cent
Same titer	48	40.7	62	64.6	56	50.0	77	80.2
One dilution different	60	50.8	32	33.3	42	37.5	19	19.8
Two dilutions different	10	8.5	2	2.1	14	12.5	0	—
Total no. of duplicates	118	100.0	96	100.0	112	100.0	96	100.0
Reproducibility[b]	91.5%		97.9%		87.5%		100.0%	

[a] See Table 2, footnote *a*.
[b] See Table 2, footnote *b*.

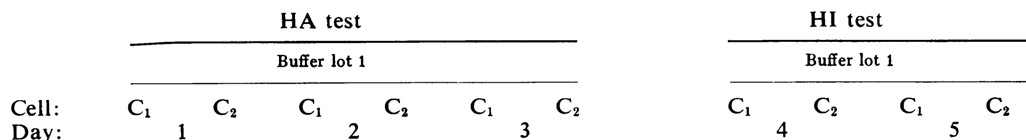

FIG. 2. *Experimental design for evaluation of standardized HA-HI tests with reovirus.*

gens. The HI tests were performed on five lots of reovirus type 1 chicken antisera, five lots of type 2, and four lots of type 3. Three normal chicken sera were used as negative controls. The negative sera were tested with all three reovirus types and were consistently <1:10. The titer range of the positive sera was 1:10 to 1:320.

Results of reovirus tests. The agreement of duplicate titers obtained on the same day for the reovirus tests is summarized in Table 6. Over 18% of the duplicate HA titers differed by two or more dilutions; hence, within-day reproducibility was 81.7%. Only one pair of duplicate HI titers differed by two or more dilutions, resulting in reproducibility of 98.2%. Within-day reproducibility of the HI test is significantly better than that of the HA test.

The agreement of duplicate titers with different red cells used for each duplicate is summarized in Table 7. The reproducibility of HA titers appears to be greater when different cells are used with duplicates than when the same cells are used (93.3% versus 81.7%). However, based on a chi-square test ($P = 0.05$), the two estimates of reproducibility are not significantly different. The effect of cell changes on the HI test also is not

significant. Apparently, the use of different cells has little or no effect on within-day reproducibility of reovirus HA and HI titers.

The day-to-day agreement of titers for the two tests in which the same cells and buffer lots were used on both duplicates of a pair is summarized in Table 8. If only the reproducibility estimates (77.5% for HA and 96.4% for HI) in Table 8 are considered, day to-day reproducibility of each test is not significantly different from within-day reproducibility. However, differences between the percentages occurring in each category of agreement are significant. That is, day-to-day variation significantly reduced the percentage of duplicates with identical titers, but only to the extent that one-dilution differences were increased. Day-to-day reproducibility of the HI test is significantly better than that of the HA test.

TABLE 6. *Within-day reproducibility of reovirus tests*

Agreement[a] of duplicate titers obtained on same day	HA		HI	
	No. of pairs	Per cent	No. of pairs	Per cent
Same titer	26	43.3	37	66.1
One dilution different	23	38.3	18	32.1
Two dilutions different	7	11.7	1	1.8
Three dilutions different	4	6.7	0	—
Total no. of pairs	60	100.0	56	100.0
Reproducibility[b]	81.7%		98.2%	

[a] Antigens and sera whose duplicate titers were negative are excluded to avoid artificially overestimating the reproducibility.

[b] Reproducibility is defined as the percentage of duplicate titers that are the same or differ by one dilution in either direction (excluding negative pairs).

TABLE 7. *Agreement of titers with cell A and cell B in reovirus tests*

Agreement[a] of duplicate titers obtained on same days using different cells	HA		HI	
	No. of pairs	Per cent	No. of pairs	Per cent
Same titer	23	38.3	33	58.9
One dilution different	33	55.0	20	35.7
Two dilutions different	4	6.7	3	5.4
Total no. of pairs	60	100.0	56	100.0
Reproducibility[b]	93.3%		94.6%	

[a] See Table 6, footnote *a*.

[b] See Table 6, footnote *b*.

TABLE 8. *Day-to-day reproducibility of reovirus tests*

Agreement[a] of duplicate titers obtained on different days	HA		HI	
	No. of pairs	Per cent	No. of pairs	Per cent
Same titer	21	26.3	22	39.3
One dilution different	41	51.2	32	57.1
Two dilutions different	18	22.5	2	3.6
Total no. of pairs	80	100.0	56	100.0
Reproducibility[b]	77.5%		96.4%	

[a] See Table 6, footnote *a*.

[b] See Table 6, footnote *b*.

DISCUSSION

The standardized HA and HI tests described here are not different in essence from the myriad of HA-HI procedures which have been used over the past two decades. The standardization is simply an effort to reduce many of the arbitrary factors of HA-HI tests to constant ones.

The standardized tests, in which a simple PBS diluent, spectrophotometrically standardized RBC suspensions, limited serum treatments, and constantly defined volumes are used, were thoroughly evaluated in the microtiter system with rigid styrene "U" plates. The HA test with adenovirus, measles, and myxovirus has a reproducibility (duplicate titers being the same or one dilution different) of 84 to 96%, and the HI test has a reproducibility of 96 to 100%. Vaccinia was also evaluated in the standardized tests, but the numbers of antigens and sera were not sufficient to obtain valid estimates of reproducibility. Reproducibility for the reovirus HA test was 78 to 93% and for the HI test was 95 to 98%. These values include equipment and human errors as major factors in within-day and day-to-day reproducibility.

There are few reports of reproducibility for HA and HI tests with which to compare these results. Simon (22) obtained standard deviations of ±0.15 for 30 adenovirus type 3 HA tests and ±0.14 for type 16. Drescher, Hennessy, and Davenport (2), using a photometric procedure for measuring the density of nonagglutinated cells at precisely timed intervals, reported a standard deviation of mean titer values of 3.5% for influenza hemagglutination. Horsfall and Tamm (6)

employed a series of fractional dilutions of myxovirus and antiserum in their HA and HI tests and found a mean deviation of ±10% in HA titrations and ±8% in HI titrations.

During the course of this investigation, we found that kaolin treatment of sera for adenovirus and measles HI tests was not routinely necessary. Schmidt, Lennette, and King (20) have reported that *Pseudomonas* filtrates are more selective in removing nonspecific inhibitors than kaolin, but that relatively few human sera contain substances inhibitory to the common adenoviruses. Zolotarskaya and Dreizin (27) showed that the normal sera of rabbits, chickens, rats, guinea pigs, and horses contain both thermolabile and thermostable inhibitors, the latter being removable by kaolin in most cases. Human sera also contained thermostable inhibitors against several of the adenovirus types, although to much lower titers than some of the animal sera. Their reported high incidence of kaolin-removable inhibitors is at variance with our findings and those of Schmidt.

In general, serum treatment methods for removal of nonspecific inhibitors of viral hemagglutination are crude and empirical. When the exact nature of these inhibitors is more fully understood, it should be possible to apply specific methods for their removal from serum specimens. The standardized HI test could then be modified to include these procedures.

The standardized HA-HI tests as described in this report reflect the simplification of HA-HI procedures as far as is possible at this time with the number of viruses involved. The standardized tests have been shown to be reliable and reproducible. Their adoption by viral diagnostic laboratories is especially encouraged in order to facilitate the performance of these tests and to render the results more comparable from one laboratory to another. Standardization is not, however, introduced to discourage research for more specific and reliable tests. On the contrary, it should prove highly useful in establishing the value of any new viral identification procedure by providing an evaluated method with which to compare the new technique.

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