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Simplified Microneutralization Test for Serotyping Adenovirus Isolates

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A simplified microneutralization procedure is described that uses an empirically determined virus challenge dose, a single dilution of antiserum, and observation of cytopathic effect to determine the adenovirus serotype. The simplified test has faster turnaround time and was 96% concordant with a confirmatory test using serial dilutions of type-specific sera. This method will find utility in high-volume serotyping work.

Adenovirus (Ad) type 4 and 7 vaccines effectively controlled Ad infections in a military population, but the virus reemerged as a major cause of acute respiratory disease when vaccine production was stopped (1, 4, 5). This prompted the Department of Defense to establish surveillance for Ad infections among trainees to determine the serotype distribution and to assess the effect of vaccine loss. Because of large numbers of Ads isolated, an efficient and rapid method for serotyping isolates was devised.

Ad serotype classification by neutralization with type-specific antisera in a microplate format has been previously described (2, 3, 6). This technique requires prior titration of viral isolates to determine the virus challenge dose and takes 3 to 7 days to complete. In this report, we describe a simplified microneutralization test using an empirically determined virus dilution and a single antiserum concentration. This research has been conducted in compliance with all applicable federal regulations governing the protection of human subjects in research.

A549 cells (American Type Culture Collection, Manassas, Va.) were maintained by monthly subculturing at a 1:4 split ratio, using Eagle's minimum essential medium in Earle's balanced salt solution, fetal bovine serum (10%), antibiotics, and amino acids (Biowhittaker, Walkersville, Md.). Prototype Ads were purchased from the American Type Culture Collection and subpassaged in A-549 cells. Type-specific rabbit immune sera to Ad types 1 to 5, 7, and 21 (Viral and Rickettsial Disease Laboratory [VRDL], California Department of Health Services, Berkeley, Calif.) were standardized to contain 20 antibody units per 10 μl. Each antibody unit neutralizes 100 50% tissue culture infective doses ($TCID_{50}$) of the prototype virus. The serotypes used in the test were those most frequently associated with respiratory disease (7).

Ad surveillance was conducted from October 1996 to June 1998 (5) among symptomatic military recruits from four training camps in the United States. Throat specimens were inoculated into A-549 cells. When 75% cytopathic effect (CPE) was observed, the cells were scraped and resuspended in culture medium, spotted, and stained with Ad-specific fluorescence-labeled monoclonal antibody (Chemicon International, Temecula, Calif.).

Simplified microneutralization. An assay medium consisting of Eagle's minimum essential medium in Earle's balanced salt solution, fetal bovine serum (10%), $NaHCO_3$ (0.09 g), L-glutamine (2 mM), penicillin (200 U/ml), streptomycin (200 μg/ml), and Fungizone (1.0 μg/ml) was used in the test. Single dilutions (10 μl) of type-specific hyperimmune rabbit sera were loaded in each well and diluted with 40 μl of assay medium. Isolates were vortexed, diluted 1:10, and loaded into designated wells at 50 μl per well. The infectivities of the isolates were assayed in a logarithmic serial dilution using 50 μl of the isolate at 1:10 dilution. The plates were incubated for 1 h at 37°C in 5% CO_2, and then 5,000 A549 cells were added to each well. The plates were further incubated for 7 days and observed after 72 h and on days 5 and 7. The serotypes were determined by inhibition of CPE in wells containing type-specific antiserum. Isolates not neutralized by any antiserum were retested at the dilution where 75% CPE was first observed in the infectivity assay. Isolates that did not type after repeat tests were considered nontypeable.

Validation. Blinded to the serotype results, VRDL validated 105 randomly selected isolates using the colorimetric microneutralization test described elsewhere (2). Serial 2-fold dilutions of the type-specific hyperimmune sera described above were used. The criterion for confirmation of a serotype was for the isolate to be neutralized by a dilution of the type-specific serum within 16-fold of the titer of the same serum required to neutralize the prototype virus. The type identification was considered confirmed when the titer of the isolate was at least 32 $TCID_{50}$ and the neutralization titer observed was within 16-fold of the titer observed when the same antiserum was tested against the homologous prototype virus.

One thousand eight hundred fourteen (53.1%) of the total 3,413 throat swabs collected were positive for Ad. Serotyping was attempted on 1,808 isolates, as 66 isolates were excluded due to possible contamination with the positive control used during culture.

The simplified typing method took approximately 3 to 7 days per test and successfully typed 1,567 (90%) of 1,742 isolates studied on the first attempt (Fig. 1). One hundred seventy-five

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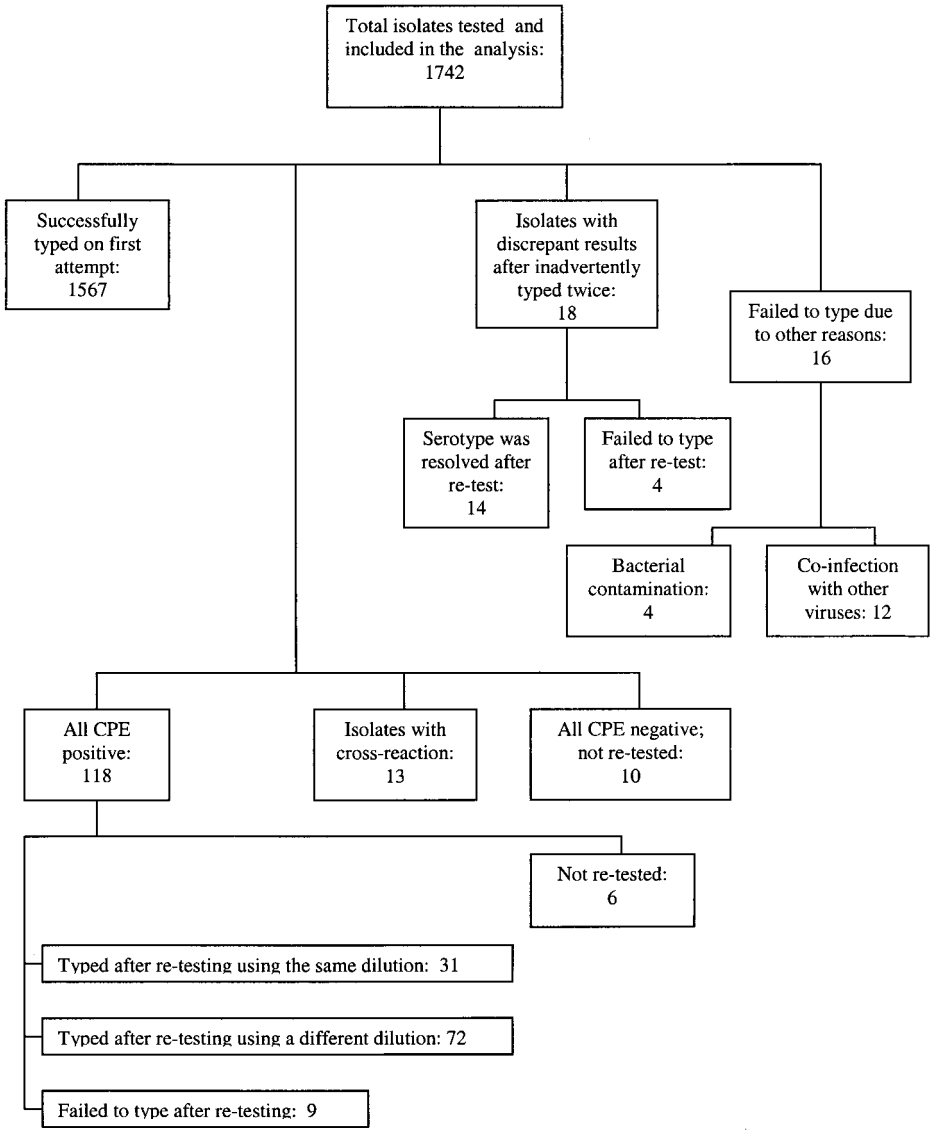

FIG. 1. Data analysis flow chart for microneutralization results.

isolates required additional attention (Fig. 1): 141 required retesting due to all serotype wells showing CPE (n = 118), two wells showing CPE (n = 13), or no wells showing CPE (n = 10); 18 had discrepant results after they were inadvertently tested twice; and 16 were contaminated with other organisms. Of the 118 isolates that were CPE positive with all antisera used, 31 typed after retesting using the same virus dilution, 72 typed after retesting at a dilution where 75% CPE was first observed in back titration, and 9 failed to type after retesting (Fig. 1). The remaining six isolates were not retested.

All 13 isolates exhibiting two serotypes were successfully typed upon retesting. The 10 isolates that were CPE negative with all antisera used were not retested. Of the samples tested, 1,697 (97.4%) were successfully typed. Overall, 4 (0.2%) of the isolates were Ad type 1, 32 (1.9%) were type 2, 142 (8.4%) were type 3, 974 (57.4%) were type 4, 407 (24.0%) were type 7, and 138 (8.1%) were type 21.

Validation results revealed agreement of the simplified microneutralization test with the standard test for 99 of 105 selected isolates. Two type 2 isolates could not be validated because of coinfection with another virus. Upon retest by the simplified procedure, two of the four discrepant isolates were in agreement with the standard (Table 1). The two remaining discrepant isolates were retested by VRDL, and serotype agreement was established. After these adjustments, a 98% concordance was observed between the results of the simplified microneutralization test and those of the standard test.

The highly concentrated antiserum used in the simplified procedure was the key factor in the successful performance of the test. Each antiserum was standardized to contain 20 antibody units that neutralize 100 $TCID_{50}$ of the prototype at a 20-fold dilution, making it possible to use an approximate test dilution, thereby cutting the assay time from 14 to 7 days.

Our results indicate that the simplified microneutralization test works well with rapidly growing, relatively high-Ad-titer viral isolates but may not perform as well for serotypes which

TABLE 1. Samples with discrepant results by the simplified microneutralization test compared to the confirmatory test

Sample	Serotype by:			
	SMT[a]	CMT[b]	Repeat SMT	Repeat CMT
1123	4	7	7	7
1257	7	4	7	7
6642	4	7	4	4
6688	2	Not typed[c]	4	4
577	2	Not typed[c]	Not done	Not done
4345	2	Not typed[c]	Not done	Not done

[a] SMT, simplified microneutralization test.
[b] CMT, confirmatory test.
[c] Not typed due to co-infection with Poliovirus I.

fail to produce high-titer virus in vitro. However, the same performance may be achieved for low-titer isolates if a higher viral challenge dose is used.

The performance characteristics of the simplified test make it useful for serotyping large numbers of isolates in laboratory-based epidemiological surveillance, outbreak investigations, and infection control in clinical settings where nosocomial infection is suspected. Its rapid turnaround time leads to cost savings, making it a valuable tool in these settings.

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