

## Serotyping of Adenoviruses Using Immune Electron Microscopy

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Immune electron microscopy may be used to determine the type specificity of several common human adenoviruses. The method described utilized equine antisera and was used to serotype adenoviruses within hours after typical cytopathic effect was observed in tissue culture. Adenovirus types 1, 2, 3, 5, 7a, 8, and 11 were isolated from clinical specimens and serotyped by serum neutralization and immune electron microscopy. Five of the seven types were identified consistently and identically by the two methods. Types 7a and 11 were indistinguishable by immune electron microscopy.

The visualization of immune reactions between viruses and antibodies has given electron microscopy a versatility which has perhaps not yet been fully appreciated. Immune electron microscopy (IEM) was first used by Anderson and Stanley to observe the behavior of tobacco mosaic virus in the presence of its specific antibody (2). The technique has since been used to demonstrate serological cross-reactivity of viruses (3), to detect small amounts of antibodies (5), to identify antigenic differences between viruses of the same type (6), and to serologically identify virions extracted directly from human tissues and feces (4, 9).

Lafferty and Oertelis were able to demonstrate distinct changes in influenza type A virus particles after incubation with homologous antiserum, whereas the same antiserum caused no demonstrable reaction when incubated with influenza B virus (8). It appeared to be possible, therefore, to utilize the electron microscope for routine serotyping of clinical isolates such as adenoviruses.

Serotyping of large groups of viruses can often be accomplished more economically and efficiently by using combinatorial pools of antisera in the serum neutralization tests (7). In this study, both combinatorial antiserum pools and IEM were employed in an attempt to serotype the more common human-associated adenoviruses.

### MATERIALS AND METHODS

**Viruses.** Prototype strains of adenovirus types 1, 2, 3, and 5 were kindly provided by Marion Cooney of the Department of Pathobiology, School of Public Health, University of Washington. Prototype strains

of adenovirus types 7a and 11 were obtained from the American Type Culture Collection. "Field" strains of adenovirus were isolated from clinical specimens over a 2-year period by the University of Washington Clinical Virology Laboratory. The clinical isolates were typed by serum neutralization in HEp-2 cell cultures, using a modification of the method of Rowe et al. (11). These strains included four isolates of adenovirus type 1, five of type 2, three of type 3, three of type 5, eleven of type 7a, one of type 8, two of type 11, and four other untyped adenoviruses. Stock viruses, all within 10 passages of the original isolate, were propagated in HEp-2 (human epithelial cell) monolayer tube cultures containing Eagle minimum essential medium. On day 1 after development of complete cytopathic effect, the cultures were subjected to three freeze-thaw cycles followed by low-speed centrifugation ( $2,000 \times g$  for 15 min) to remove cellular debris. All strains were coded and tested by IEM in duplicate without prior knowledge of the serum neutralization test results.

**Antisera.** Antisera to adenovirus types 1, 2, 3, 5, 7a, 8, and 11 were obtained from the Reference Reagents Division of the Center for Disease Control, Atlanta, Georgia. The neutralizing antibody titers of the sera, as determined by the Center for Disease Control, are listed in Table 1. Two different combinations of four antiserum pools were constructed for use in IEM so that the final dilution of each homotypic antiserum in each pool was 1:20 (Table 2). The sera were stored at  $-20^{\circ}\text{C}$  until tests were performed. Fetal calf serum was diluted 1:20 and used as a serum control in the IEM assay.

**IEM.** A pipette was used to deliver 0.05 ml of antiserum from each pool to separate wells of a U-bottom microtiter plate. An equal volume of virus suspension was pipetted into each antiserum-containing well and mixed by gentle agitation. The plate was then covered and incubated at  $35^{\circ}\text{C}$  for 1 h. After incubation, two 200-mesh copper grids (previously coated with 2% parlodion plastic and carbonized)

TABLE 1. Homologous neutralizing antibody titers of adenovirus equine antisera in rhesus monkey kidney tissue culture<sup>a</sup>

Antiserum type	Homologous titer <sup>b</sup>
1	320
2	640
3	640
5	1,280
7a	640
8	160
11	160

<sup>a</sup>Data provided courtesy of Licensure and Proficiency Testing Branch, Center for Disease Control, Atlanta, Ga.

<sup>b</sup>Reciprocal of serum dilution; type 11 antiserum also had a heterologous titer of 1:10 against type 7a prototype virus.

TABLE 2. Composition of two pooling schemes employed for identification of seven adenovirus types

Pool	Scheme	Antiserum to indicated adenovirus type			
		1	7a	8	11
A	1	1	7a	8	11
B		2	7a		
C		3		8	
D		5			11
A	2	1	2	3	5
B		2	8	7a	
C		3	7a	11	
D		5	8	11	

were submerged into each well, immediately removed, and stained with 2% phosphotungstic acid, without prior blotting. The grids were air dried and then examined on a Carl Zeiss EM 9S electron microscope. If aggregates containing three or more adenovirus particles were observed, it was inferred that homologous antiserum was present in the pool and a positive reaction was recorded, whereas random distribution of particles on the grid was recorded as a negative reaction.

## RESULTS

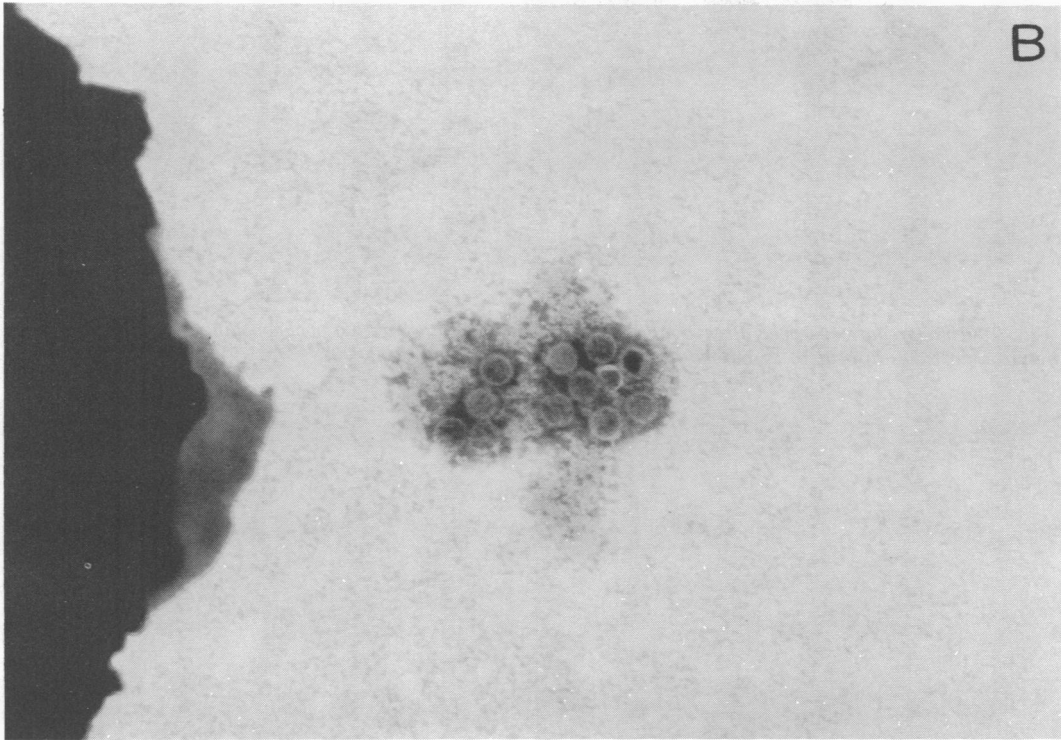
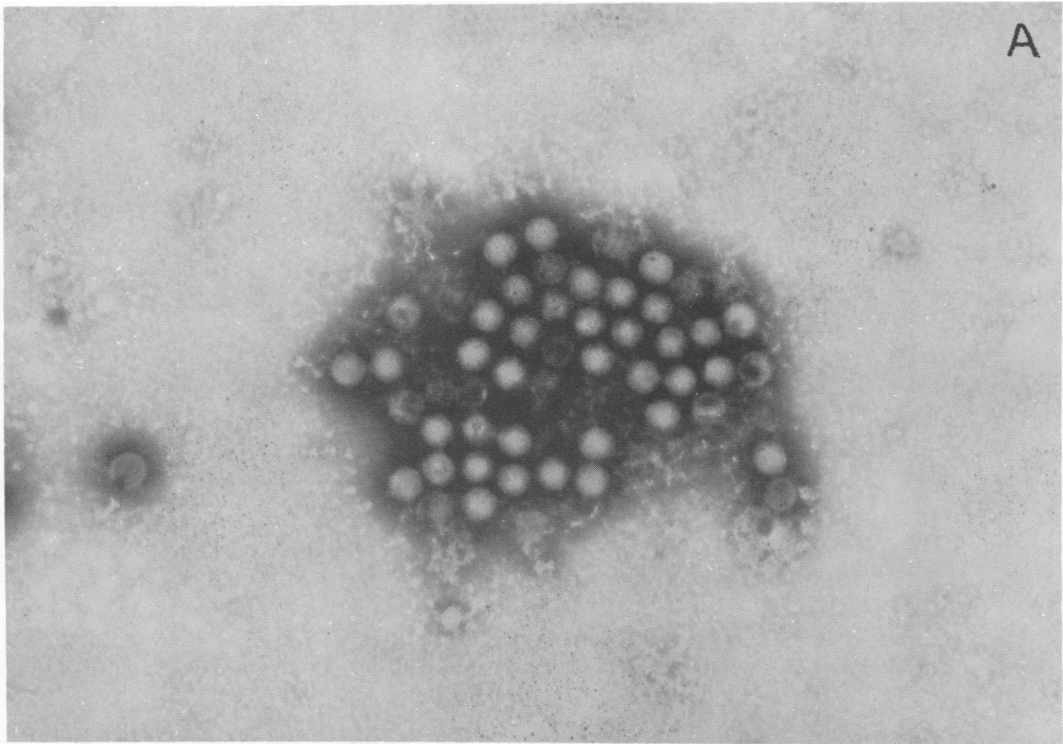
Seven distinct reaction patterns were expected in the IEM assay; however, only five of these were observed, corresponding to adenovirus types 1, 2, 3, 5, and 8. The results of both serum neutralization and IEM tests were in complete agreement for all of these strains whether they were prototypes or clinical isolates, and regardless of which pool scheme was used. Aggregates observed in positive reactions varied in size from 3 to 60 or more virus particles and contained complete virions, virus particles which appeared to be empty, or mixtures of

both (Fig. 1), whereas negative reactions uniformly showed no aggregation. Antisera were not pretreated to remove large proteins or other nonspecific debris, and as a result virus particles were not always contiguous within an aggregate as reported by Almeida and Waterson (1), but rather appeared to be embedded in a proteinaceous web (Fig. 2). No aggregates were observed in the presence of fetal calf serum.

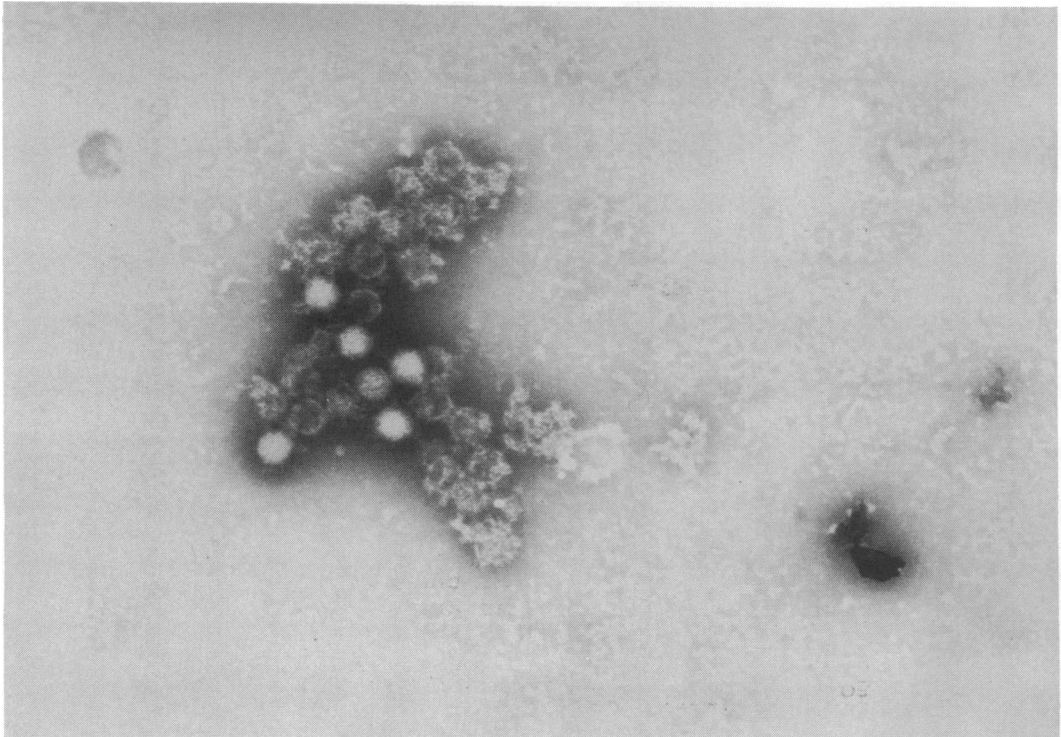
No differences in the ease of detection of aggregates were apparent when 10 clinical isolates were tested after two or three passages of the original isolate as compared to higher passage levels. The infectivity titers of the samples were determined in HEp-2 cell cultures and were found to range between  $10^2$  and  $10^4$  mean tissue culture infectious doses per ml. Therefore, it appears that the primary requisite for successful IEM application in this system was the development of complete cytopathic effect, regardless of passage level or infectivity titer.

An unexpected reaction pattern in which aggregates appeared in three of four antiserum pools when attempts were made to serotype adenovirus types 7a and 11 by IEM was repeatedly observed. This cross-reactivity was found to occur consistently with the prototype strains as well as with all of the clinical isolates. The use of either of the two pooling schemes yielded similar results; adenovirus types 7a and 11 formed aggregates in the presence of both 7a and 11 antisera, regardless of the type specificity of the other antisera in the pool.

In an attempt to diminish this apparent two-way cross-reaction, both type 7a and type 11 antisera were titrated against the prototype strains of 7a and 11 as well as two clinical isolates of adenovirus type 11 and three clinical isolates of adenovirus type 7a using IEM. None of the type 7a adenoviruses were seen to form aggregates in the presence of type 11 antiserum diluted 1:160 or greater; however, all type 7a isolates continued to form aggregates when incubated with type 7a antiserum diluted 1:640 (Fig. 3A). One clinical isolate of adenovirus type 11 formed aggregates with type 7a antiserum at a dilution of 1:320, although it ceased to react with homotypic antiserum at the 1:160 dilution; the other clinical isolate reacted only with the homotypic antiserum, but at a relatively low dilution. The type 11 prototype reacted equally well with type 7a and type 11 antisera (Fig. 3B). These findings contrast with the heterologous reactions observed with standard neutralization tests. In these tests, type 11 antiserum was found to have a heterologous titer to type 7a prototype of only 1:10, and no heterologous reaction was noted between type 7a antiserum and type 11 prototype.



**FIG. 1.** Appearance of aggregates observed after incubation of adenoviruses with pools containing homologous antiserum. Aggregates consist of complete virions, virus particles which appear to be empty, or combinations of both. (A) Adenovirus type 7a; (B) adenovirus type 11.  $\times 60,000$ .



**FIG. 2.** An aggregate observed after incubation of adenovirus type 3 with a pool containing type 3 antiserum. The virus particles appear to be embedded in a proteinaceous web.  $\times 60,000$ .

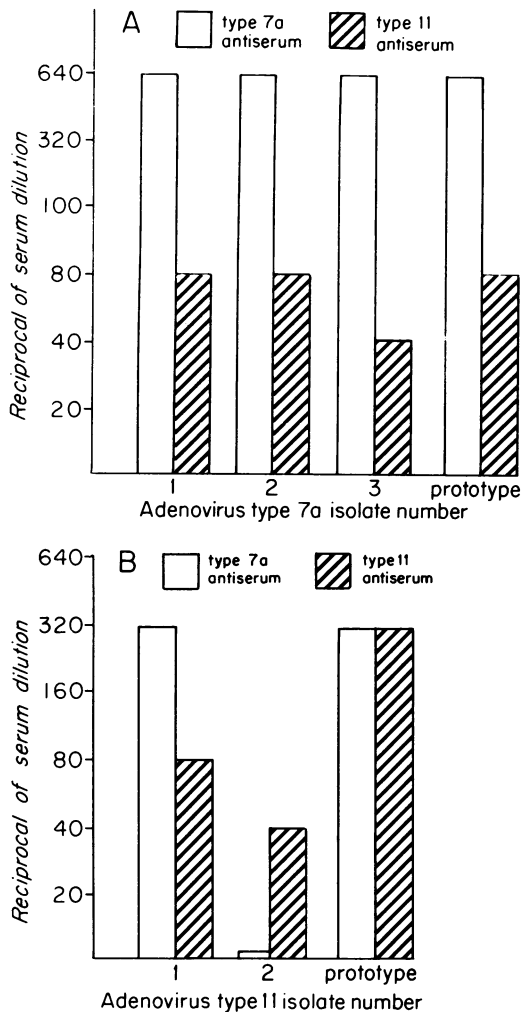


FIG. 3. IEM reactions of type 7a and type 11 adenovirus antisera with prototype and clinical isolates of adenovirus type 7a and type 11. (A) Adenovirus type 7a; (B) adenovirus type 11.

### DISCUSSION

The IEM typing assay described was designed as a practical method for the rapid serotyping of adenoviruses from tissue culture. Pooling schemes were employed to make the assay more efficient, and low serum dilutions were used to detect all possible reactions. Ultracentrifugation caused nonspecific aggregation of adenovirus particles and was therefore avoided.

Positive reactions were generally characterized by an abundance of aggregates and very few isolated particles, in contrast to the negative reactions which contained no aggregates, or an occasional pair of particles in close proxim-

ity. With the exception of types 7a and 11, all clinical isolates and prototypes were identified readily when coded and interpreted in "blind" studies.

It was not possible to identify type 7a or type 11 adenoviruses by IEM. Cross-reactions between types 7a and 11 appear to be common and may be the result of antigenic similarities which have been previously described (10).

This study involved only a small number of adenovirus types, and therefore generalizations must be made with caution. IEM may have potential as a routine typing system for other virus groups, and its application deserves further investigation.

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