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Comparison of Neutralization and DNA Restriction Enzyme Methods for Typing Clinical Isolates of Human Adenovirus

KENNETH H. FIFE,[1]* RHODA ASHLEY,[2,3] ANTHONY F. SHIELDS,[4,5] DEBORAH SALTER,[2,3] JOEL D. MEYERS,[4,5] AND LAWRENCE COREY[2,3,4,6]

Departments of Medicine and Microbiology and Immunology, Indiana University School of Medicine, Indianapolis, Indiana 46223[1]; Departments of Laboratory Medicine,[2] Medicine,[4] and Pediatrics,[6] University of Washington School of Medicine, Fred Hutchinson Cancer Research Center[5]; and Children's Orthopedic Hospital Medical Center,[3] Seattle, Washington 98105

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Sixty-five adenovirus isolates collected over a 3.5-year period were typed by both standard microneutralization techniques and restriction endonuclease digestion of viral DNA. Of the 65 isolates, 47 (72.3%) representing six adenovirus types could be typed by microneutralization. Eighteen isolates demonstrated partial neutralization with standard antisera to two or more adenovirus serotypes and thus could not be definitively typed. DNA analysis permitted typing of 64 of the 65 isolates (98.5%) (including four isolates which contained mixtures of two adenovirus types), and 12 different types were identified. Neutralization and DNA typing disagreed for five isolates, and in each case, digestion with multiple restriction endonucleases and DNA hybridization studies were consistent with the type assigned by DNA analysis. In addition, the DNA analysis method allowed the identification of genomic variants (genome types) of five adenovirus types. We conclude that typing clinical isolates of adenovirus by restriction endonuclease digestion of viral DNA can be done rapidly, provides additional epidemiological and typing information, and provides fewer ambiguous results than does typing by neutralization.

Adenoviruses are important causes of ocular, respiratory, gastrointestinal, and genitourinary diseases in both adults and children and are being increasingly recognized as important pathogens of immunosuppressed patients (7, 9, 11, 15, 16, 20, 22, 26, 27). To date, 41 types or species of human adenovirus (5) have been described. Adenoviruses have been grouped by several systems based on a variety of biological and biochemical parameters which are shared by viruses of different species. The most widely accepted system places the known serotypes into seven groups or subgenera (A through G) based on the molecular weights of the major capsid proteins and on DNA sequence homology (8, 24). Traditionally, typing of adenoviruses has been performed by reacting defined antisera (made against prototype viruses) with the isolate to be typed in a neutralization assay. The reaction in a neutralization assay has been accepted by the International Committee on Taxonomy of Viruses as the definition of an adenovirus type (or species) (25). However, neutralization assays are time-consuming, sometimes difficult to interpret, and absolutely dependent upon the availability and quality of type-specific antisera. In an effort to improve the speed and accuracy of adenovirus typing, we have adapted the technique of restriction endonuclease digestion of adenovirus DNA in a way which permits rapid analysis of large numbers of isolates. We report here a comparison of neutralization assay and DNA analysis methods and illustrate several unique advantages of the latter system.

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* Corresponding author.

MATERIALS AND METHODS

Viral isolation. Viral isolates were obtained by inoculation of clinical specimens into duplicate tubes of either human embryonic tonsil cells or primary cynomolgus monkey kidney, HEp-2, diploid fibroblast, and human embryonic kidney cells. Adenovirus was identified by typical cytopathic effect and confirmed by an indirect immunofluorescent assay with a monoclonal antibody to the hexon antigen of adenovirus (4). Viral stocks of each of the adenovirus isolates were then grown in HEp-2 cells and frozen at −70°C pending further analysis.

Neutralization assay. For typing by microneutralization, adenovirus stocks were grown in HEp-2 cells, and titers were determined in microtiter plates containing confluent HEp-2 cells. For the neutralization assay, 100 tissue culture infectious doses of each stock virus were incubated with 0.025 ml of antiserum to adenovirus types 1 through 33 obtained from either the Centers for Disease Control, Atlanta, Ga., or the American Type Culture Collection, Rockville, Md. Subsequently, all isolates were also tested for neutralization with antisera to adenovirus type 34 (Ad34) and Ad35 (kindly supplied by John Hierholtzer and Gary Noble of the Centers for Disease Control). The adenovirus type was determined by the antiserum, which completely inhibited viral growth after 72 h of incubation. Isolates which exhibited neutralization with more than one antiserum were reassayed. Isolates which were neutralized by more than one antiserum on repeated assays were designated as nontypable. The adenovirus isolations were performed at either the University of Washington Diagnostic Virology Laboratory or the Fred Hutchinson Cancer Research Center in Seattle, Wash. All neutralization assays were performed at the University of Washington Laboratory located at the

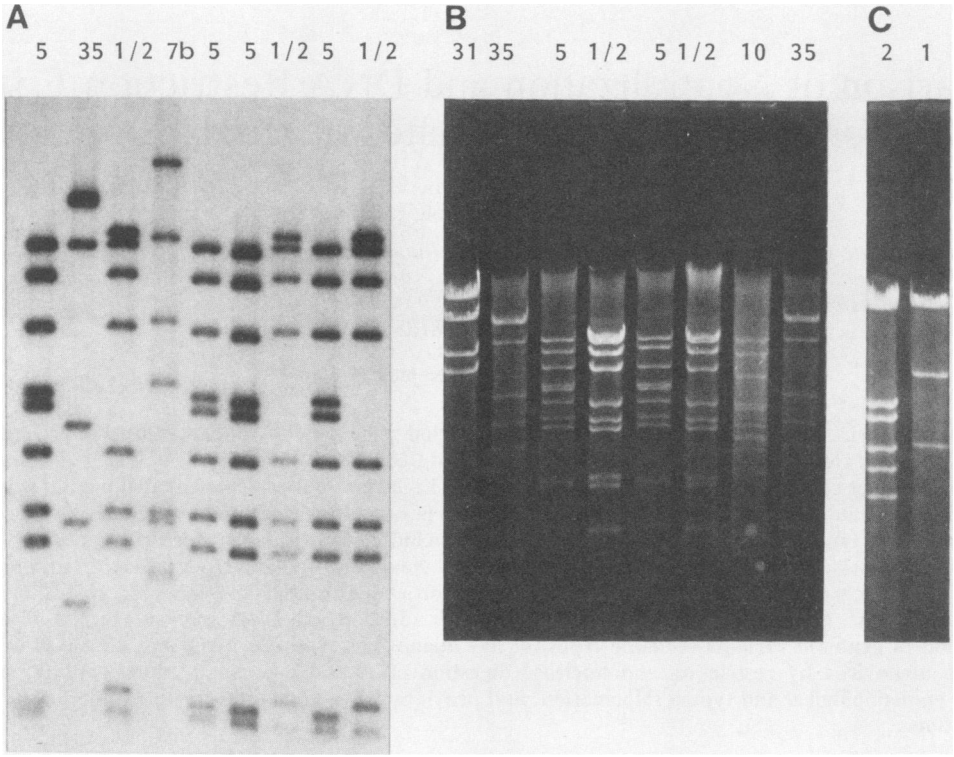

FIG. 1. Representative restriction endonuclease digestion patterns with either ^{32}P-labeled DNA (A) or unlabeled, ethidium bromide-stained DNA (B and C). The numbers above the lanes refer to the type assignments. Panels A and B were digested with *Sma*I. Several lanes are labeled 1/2 to indicate that Ad1 and Ad2 have identical digestion patterns and cannot be distinguished. Panel C shows the two DNAs labeled 1/2 in panel B digested with *Eco*RI allowing Ad1 to be distinguished from Ad2, as indicated.

Children's Orthopedic Hospital Medical Center, Seattle, Wash.

DNA extraction. Harvests of clinical isolates or stock viruses (obtained from the American Type Culture Collection) were inoculated onto confluent monolayers of HeLa cells in 24-well microwell plates (Linbro; Flow Laboratories, McLean, Va.) which have a growth area of about 1 cm^2 per well. Cells were observed for cytopathic changes; when extensive cytopathic change was evident, DNA was extracted by a modification of the Hirt procedure (10, 17). After removal of the medium and a phosphate-buffered saline wash, cells were lysed in the microwell plate by the addition of 300 μl of 0.6% sodium dodecyl sulfate–10 mM EDTA and incubated with protease (type XIV; Sigma Chemical Co., St. Louis, Mo.) at a concentration of 275 μg/ml. High-molecular-weight (cellular) DNA was then precipitated by the addition of NaCl to a concentration of 1.0 M and incubation at 4°C for 4 h. The supernatant fluid (containing predominantly viral DNA) was treated with 10 μg of ribonuclease A (Sigma) and phenol extracted, and the DNA was concentrated by precipitation with 2 volumes of ethanol. The extraction procedure was readily performed in a 1.5-ml microcentrifuge tube. In most cases, the DNA yield from one well was sufficient for 5 to 10 restriction endonuclease digestions. In some cases, viral DNA was radiolabeled by infecting cells in medium without sodium phosphate and adding 50 μCi of $^{32}P_i$ (New England Nuclear Corp., Boston, Mass.) to each well 16 h after infection.

DNA purification. For hybridization experiments, purified adenovirus DNA was used. Infected cells were lysed in 2% sodium deoxycholate (Sigma) and 0.01% trypsin (Flow Laboratories), and virions were purified by banding twice in cesium chloride equilibrium density gradients with a mean density of 1.34 g/cm^3 (1). Virions were disrupted in 0.6% sodium dodecyl sulfate and 1 mg of protease (Sigma type XIV) per ml, and the viral DNA was recovered by ethanol precipitation after phenol extraction (12).

Gel electrophoresis. All restriction endonucleases were purchased from Bethesda Research Laboratories, Gaithersburg, Md., and digestions were carried out under conditions specified by the supplier. After digestion, samples were loaded onto 0.7% agarose gels (5 by 7.5 cm) and electrophoresed at 80 V for 75 min in a 50 mM Tris–borate–EDTA buffer (pH 8.0) (14). Gels containing unlabeled DNA were run in the presence of 0.25 μg of ethidium bromide per ml. The bands were visualized under long-wave UV light and photographed on Polaroid type 57 film with a Wratten 23A filter. Gels containing radiolabeled DNA were run in a larger apparatus (20 by 20 cm) at 60 V for 18 h, then dried, and exposed to Kodak XAR-5 medical X-ray film with Cronex Lightning Plus intensifying screens for autoradiography. The restriction endonuclease digestions were performed at Indiana University without knowledge of the neutralization assay typing results.

DNA typing assay. DNA from each isolate was initially digested with the restriction endonuclease *Sma*I, and type assignments were made by comparing the resulting pattern with patterns of prototype viruses either run in parallel experiments or taken from the literature (e.g., reference 22). Some typical patterns are shown in Fig. 1 with radiolabeled (1A) and unlabeled (1B) DNA. For adenovirus types in which the *Sma*I patterns were identical (e.g., Ad1 and Ad2), additional digestions with *Eco*RI were performed (Fig. 1C).

DNA hybridization. Appropriate restriction endonuclease

fragments were separated by electrophoresis on 0.7% agarose gels. After locating the fragments by ethidium bromide staining, the fragments were transferred to replicate nitrocellulose membranes by the method of Southern (19) as modified by Smith and Summers (18). Probe DNA was labeled by nick translation with a commercial kit (Bethesda Research Laboratories) and [α-^{32}P]dCTP (New England Nuclear Corp.). Transfers were hybridized with probe DNA at 68°C for 20 h. After being washed, the nitrocellulose membranes were dried and exposed to X-ray film as above.

RESULTS

Neutralization assay. All of the adenoviruses isolated in the University of Washington Diagnostic Virology Laboratory between January 1980 and July 1983 were typed by both neutralization assay and DNA analysis. Adenovirus isolates were obtained from 66 patients. Duplicate isolates obtained from the same patient (in all cases, duplicate isolates were of the same type) were not included in the totals. One isolate grew very poorly and could not be further analyzed. Type assignments (as determined by neutralization assay), sites of isolation, and patient populations for the remaining 65 isolates are summarized in Table 1. Forty-seven isolates (72.3%) could be assigned to a type by microneutralization. For 6 of these 47 isolates (9.2%), initial testing demonstrated partial neutralization with more than one adenovirus antiserum, and repeat testing was needed to ascertain which type-specific antiserum gave the most reproducible endpoint. Overall, 18 isolates could not be definitively assigned by microneutralization testing to a single adenovirus type. For each of these isolates, equal neutralization with two or more of the type-specific antisera was noted even with repeat testing.

TABLE 1. Neutralization assay

Adenovirus subgenus and type	No. of isolates from indicated site[a] in following patients:											
	Bone marrow transplant				Chronic adenopathy			Immunocompetent				
	R	GI	U	V	GI	U	LN	E	R	GI	U	V
A												
12												
31												
B												
3												
7	1								1	1		
11			3									
34												
35												
C												
1	5	4		1	1	1				1		
2	3			1			2	1	2	1		1
5	7	1	5	1						1		
D												
10												
19					1			1				
E												
4												
Nontypable		1	9			2			1	3	2	

[a] Abbreviations: R, respiratory tract; GI, gastrointestinal tract; U, urinary tract; V, visceral organ; LN, lymph node; E, eye.

TABLE 2. DNA analysis

Adenovirus subgenus and type	No. of isolates from indicated site[a] in following patients:											
	Bone marrow transplant				Chronic adenopathy			Immunocompetent				
	R	GI	U	V	GI	U	LN	E	R	GI	U	V
A												
12										1		
31		1								1	1	
B												
3			1								1	
7	1								2	1		
11			1									
34			1									
35			8			2						
C												
1	5	4	1	1					1	1		
2	3[b]			1		1	2	2[c]	1[c]	1		1
5	7	1	5	1						1		
D												
10										1		
19												
E												
4					1							
Nontypable					1							

[a] Abbreviations: R, respiratory tract: GI, gastrointestinal tract; U, urinary tract; V, visceral organ; LN, lymph node; E, eye.

[b] One isolate contained a mixture of a genome type of Ad2 and a genome type of Ad5.

[c] Each isolate contained a mixture of Ad2 and Ad5.

Adenoviruses were isolated most commonly from patients undergoing bone marrow transplantation, from homosexual men with chronic generalized lymphadenopathy, and from children with lower respiratory or gastrointestinal illnesses. Of interest was that 12 of 20 adenoviruses isolated from the urine of bone marrow transplant patients and homosexual men with lymphadenopathy demonstrated partial neutralization with antisera to Ad11, Ad34, or Ad35, suggesting that they belonged to subgenus B. However, on repeated testing only 3 of these 12 isolates could be assigned to one of these three subgenus B types.

DNA analysis. The 65 clinical isolates were subjected to analysis by restriction endonuclease digestion of viral DNA, and the results are shown in Table 2. Of the 65 isolates, 60 (92.3%) could be clearly assigned a type. Five isolates yielded patterns which were not typical of previously seen adenoviruses. Four of these five isolates appeared to be mixtures of two adenovirus types, and the two types present were tentatively assigned by inspection of the patterns (Fig. 2). Three of these isolates were mixtures of Ad2 and Ad5, while one contained a variant of Ad2 (genome type 2b) as well as Ad5. One isolate, a rectal swab culture from a man with chronic generalized lymphadenopathy, had a DNA pattern different from those of any prototype virus or previously described variant. This isolate was neutralized by antiserum to Ad19 but had a cleavage pattern clearly different from that of prototype Ad19 and the variant Ad19a. Figure 3 shows the digestion pattern of the DNA of this isolate along with several subgenus D prototypes. This isolate actually had more bands in common with Ad10 than with Ad19, but it was not neutralized by antiserum to Ad10.

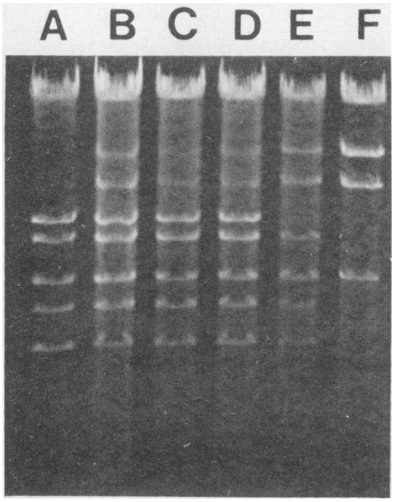

FIG. 2. *Eco*RI digest of suspected adenovirus mixtures. Lanes contain: A, a prototype Ad2 DNA; F, a prototype Ad5 DNA; B through E, DNA from the four isolates suspected of being mixtures. Note that the isolate in lane E is missing one of the Ad2 bands, typical of genome type 2b (6).

Eight isolates which were neutralized with antiserum to Ad1 or Ad2 had variant cleavage patterns which have been mapped and reported as new genome types of these viruses (6). Three additional isolates which were not typable by neutralization assay also had variant cleavage patterns but again could be easily related to prototype viruses. Two were tentatively designated as genome types of Ad3 (22) and one as a genome type of Ad12. In addition, four isolates (three that were typed by neutralization assay as Ad7 and one which was not typable) were found to be genome type 7b (23).

Comparison of the two methods. The results of the two methods are compared in Table 3. There were discrepancies between neutralization assay and DNA analysis type assignments for five isolates. For two of these isolates, the subgenus designation differed between the two methods. One isolate was neutralized by antiserum to Ad1 (subgenus C) but had a DNA digestion pattern identical to that of Ad4 (subgenus E), and one isolate was neutralized by antiserum to Ad19 (subgenus D) and found on DNA analysis to contain both Ad2 and Ad5 (both subgenus C). The first isolate was further analyzed by DNA hybridization studies. DNA from this isolate (neutralized as Ad1, DNA pattern of Ad4) was digested with restriction endonucleases and run on a gel with prototype Ad1 and Ad4 DNA. DNA from this gel was transferred to nitrocellulose membranes and hybridized to either Ad1 or Ad4 DNA which had been radiolabeled by nick translation. The clinical isolate had a hybridization pattern identical to that of the prototype Ad4 (data not shown). The isolate neutralized by antiserum to Ad19 and found on DNA analysis to be a mixture of Ad2 and Ad5 was not investigated further (this isolate is shown in Fig. 2, lane C).

Three isolates showed discrepancies between the two typing methods such that they were assigned to the same subgenus but to different types within the subgenus. One virus was neutralized by antiserum to Ad2 but had a DNA digestion pattern like that of Ad1. Cleavage of viral DNA from this isolate with the restriction endonucleases *Eco*RI, *Hin*dIII, *Kpn*I, and *Hpa*I revealed patterns identical to those of prototype Ad1 and different from those of Ad2. There are a total of 11 cleavage sites for these four enzymes which differ between Ad1 and Ad2 (2).

Two isolates were neutralized with antiserum to Ad11 but had DNA digestion patterns which most closely resembled those of Ad35. These isolates are shown in Fig. 4 with the other related subgenus B isolates. Although most of these

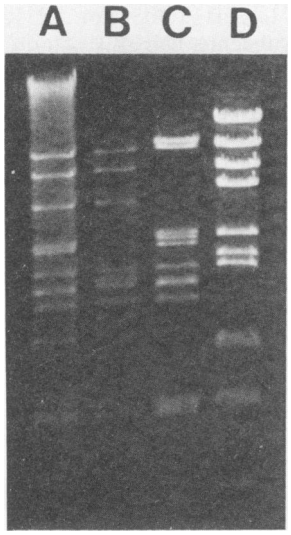

FIG. 3. *Sma*I digestion of the DNA of the isolate which could not be matched to DNA patterns of prototype viruses and comparison with related subgenus D viruses. Lanes: A, Ad10; B, unknown isolate; C, Ad19; D, Ad25.

TABLE 3. Method comparison

DNA result (adenovirus subgenus and type)	Neutralization results for following adenovirus subgenus and type (no. of isolates)													
	A		B					C			D		E 4	NT[a]
	12	31	3	7	11	34	35	1	2	5	10	19		
A														
12[b]														1
31														3
B														
3[b]														2
7[b]				3										1
11					1									
34														1
35					2									8
C														
1[b]								11	1					1
2[b]									11[c]			1[c]		
5										15				
D														
10														1
19														
E 4								1						
NT												1		

[a] NT, Not typable.

[b] Includes one or more variants with slightly altered cleavage patterns (genome types).

[c] Includes a total of four isolates which contained a mixture of two viruses by DNA analysis.

isolates appear to be more closely related to Ad34 or Ad35 than to Ad11, digestion of these isolates with additional restriction endonucleases showed them to be a rather heterogeneous group. Further characterization of these isolates is currently underway. Initial experiments suggest that some of these isolates are previously unrecognized genome types of these species.

DISCUSSION

In this series of 65 sequential clinical adenovirus isolates from a clinical virology laboratory, adenovirus typing by DNA analysis appeared to be more accurate and provided more information than standard microneutralization methods. Using the microneutralization assay, we identified 47 isolates which included six different adenovirus types. Of the typable isolates, 6 (9.2%) had microneutralization assay results that required repeating one or more times to determine the adenovirus type definitively. Of 65 isolates, 18 (27.7%) were not typable by microneutralization testing, demonstrating partial neutralization with several antisera even on repeat analysis. With DNA analysis, 64 isolates were typed, and these included 12 different adenovirus types. Only 1 of 65 isolates (1.5%) could not be readily assigned to a type, and repeat assays were seldom needed.

Two isolates gave markedly different results in the microneutralization and DNA analysis assays. In these cases of disagreement in subgenus assignments, both digestion with multiple restriction enzymes and DNA hybridization studies indicated that the type assignment made by DNA analysis was correct. In theory, a recombination event could produce a virus which neutralizes with one type of antiserum while containing most of the DNA sequences of another type. While this possibility cannot be totally excluded, there is generally less than 20% DNA sequence homology between viruses of different adenovirus subgenera, so homologous recombination would be very unlikely to occur.

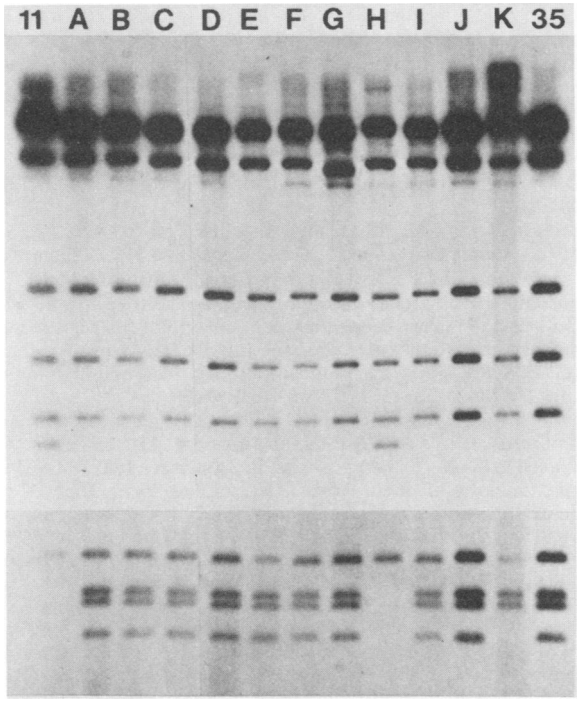

FIG. 4. *Sma*I digest of Ad11, Ad34, and Ad35 isolates. The far left lane contains prototype Ad11 DNA, and the far right lane contains prototype Ad35 DNA. The lanes labeled A through K contain 11 of the clinical isolates (one was not available for this analysis). The isolate in lane I was neutralized by antisera to Ad34 and Ad35; all others were neutralized by antiserum to Ad11, and all but the isolates in lanes A, C, and H were also neutralized by antisera to Ad34 or to Ad35, or to both. To show the smaller fragments, the lower section of the figure is taken from a longer exposure of the same autoradiograph.

The initial neutralization assays were done with a bank of antisera to Ad1 through Ad33. With these antisera, 11 isolates gave microneutralization assay and DNA analysis results which assigned the isolates to different members of the same subgenus. Ten subgenus B isolates which initially neutralized with antiserum to Ad11 had DNA digestion patterns like those of Ad34 or Ad35. Subsequent analysis of these isolates with Ad34 and Ad35 antisera revealed that eight were also neutralized by one of these sera. Thus, eight of these isolates were not typable, and two continued to show disagreement. One of the advantages of the DNA analysis technique is that it permits identification of new types (or of types not represented in the laboratory antiserum bank).

One isolate which was neutralized by antiserum to Ad2 had cleavage patterns with multiple restriction endonucleases identical to those of Ad1. Most type-specific antigenic determinants of adenoviruses involving neutralization are thought to be contained on the hexon protein (13) which is encoded in the region from 50 to 59 map units on the adenovirus genetic map. Ad1 and Ad2 have few cleavage site differences in this region. The *Eco*RI site at 58.5 map units which is present in Ad2 but not Ad1 was not present in this isolate, but this was the only distinguishing site in the hexon gene region. Thus, we cannot totally exclude the possibility that this virus was a recombinant between Ad1 and Ad2 which contained most of the Ad1 genome but part of the Ad2 hexon gene.

Four isolates were found to be mixtures of two different adenovirus types. None of these isolates was suspected of being a mixture by neutralization assay. Three of the four mixed isolates were initially cultivated within a 1-month period and contained the same two types (Ad2 and Ad5). Thus, the possibility of a laboratory contaminant cannot be excluded. The fourth isolate was from a different time of cultivation, and reinoculation of the original specimen again yielded two distinct viruses, suggesting that the patient was shedding both viruses. It is thus possible to detect mixed infections by the DNA analysis method as long as both types are present in similar proportions. Mixed infections should also be detected in a neutralization assay, but such results might be difficult to recognize without repeat testing.

It was of interest that nine isolates had DNA patterns which differed from those of prototype viruses and were thus identified as previously undescribed genome types (6). Six additional isolates had variant patterns which were compatible with those of previously described genome types. Genome types have been important both in epidemiologic investigations (3, 20, 22) and as correlates with pathogenicity (22, 23). This observation identifies a feature which is both an advantage and a disadvantage of the DNA analysis method of typing. While DNA analysis allows for the identification of genomic variants of known adenovirus types, such variation may be more common than previously realized so that specific type assignment may be somewhat difficult by inspection of digestion patterns alone. In cases in which digestion patterns do not match with those of known

types and cannot be explained by changes in one or two cleavage sites, supplementary information from a neutralization assay is needed. This may be especially important when characterizing isolates from subgenus D because this subgenus contains a large number of types, and differences in cleavage patterns with a limited number of restriction endonucleases may be less distinctive. However, as inspection of the digestion pattern nearly always allows subgenus determination, only a limited number of neutralization assays would need to be done. Conversely, because type assignments are being made on the basis of one or two digestion patterns, some genome types may be missed.

Although the DNA analysis method uses techniques which may be unfamiliar to many diagnostic virology laboratories, it is easily learned. The materials used are readily available and are no more expensive than antisera. The equipment required is also relatively inexpensive, with the exception of the photography equipment. The amount of time required to perform the DNA analysis is less than that required for neutralization assays. In fact, DNA analysis can be performed on the contents of an infected culture tube (K. Fife, unpublished observations) and could be performed on the primary isolate or a first subpassage. Several laboratories have been using DNA analysis of adenovirus isolates to supplement serologic data, but the methods used have been difficult to apply in clinical laboratories (11, 21, 24). The method which we have adopted can be used to type relatively large numbers of isolates rapidly.

While the neutralization assay is felt to be the standard technique for typing of adenoviruses (25), we have shown that rapid DNA analysis as performed in our study can also accurately type adenoviruses and can provide a great deal of information about an isolate that cannot be obtained in a neutralization assay. We feel that these two methods provide important cross-checks on one another.

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