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Comprehensive Detection and Serotyping of Human Adenoviruses by PCR and Sequencing

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Human adenoviruses are common pathogens associated with many diseases, including respiratory, gastrointestinal, and ocular infections. Because they are now being increasingly recognized as agents of life-threatening disseminated infection in immunocompromised patients, robust and sensitive laboratory detection methods are needed for their rapid diagnosis. We describe here a PCR assay using a single primer pair, targeting a region of the hexon gene containing hypervariable region 7, that can detect all known human adenovirus serotypes and allows for serotype determination through the analysis of the nucleotide sequence. This comprehensive assay has proven effective for diagnosing adenoviruses at the serotype level in a broad range of patient specimens, including conjunctival, nasopharyngeal, stool, blood, and urine specimens.

Human adenoviruses have long been recognized as pathogens, causing a broad spectrum of diseases, including upper and lower respiratory tract infections, gastroenteritis, conjunctivitis, and keratoconjunctivitis (27, 34). Adenovirus infection is being increasingly associated with severe and potentially fatal presentations, such as pneumonia, severe hemorrhagic cystitis, hepatitis, and disseminated infection, in immunodeficient patients, including bone marrow and solid organ transplant recipients and patients with AIDS (2, 8, 12, 17, 19, 29, 34, 38). Although treatment modalities remain limited (8, 12), there have been encouraging reports on the efficacy of cidofovir, which may herald the advent of antiviral drugs more active against adenoviruses (12, 26, 38).

The increased recognition of severe adenovirus infection in immunocompromised hosts and the possibility of therapeutic intervention call for sensitive and rapid laboratory diagnosis. In this regard, conventional methods, such as isolation in cell culture, direct detection by immunospecific methods, or visualization by electron microscopy, are lacking in rapidity or sensitivity. Consequently, detection by nucleic acid testing such as PCR has been proposed to address these deficits (17, 19, 38). Accordingly, several PCR-based detection tests that target genome regions conserved among adenovirus serotypes and their clinical applications have been described (see, for example, references 6, 14, 16, 18, 20, and 25).

The classification of adenoviruses into serotypes is based on neutralization assays. To date, 51 established or candidate serotypes have been described (15, 27, 33, 34). In turn, these serotypes are further classified into six species, A to F, based on their ability to agglutinate various types of erythrocytes (33, 34). The biological soundness of this classification is underlined by the observations that other viral properties, namely, antigenic relationship, oncogenicity, fiber length, the percent DNA homology and G+C content, the cleavage patterns by SmaI, and the molecular weights of some proteins, are generally in concordance within species (34). There is a growing clinical interest in serotype determination of clinical isolates since it is becoming increasingly clear that specific serotypes are associated with manifestation and severity of the disease presentation (1, 3, 4, 5, 21, 22, 27, 30, 31, 34, 35, 38). Determination of adenovirus type by virus isolation, followed by neutralization tests with type-specific sera, may take several weeks (34), which limits the clinical value of this approach, especially in the management of immunocompromised patients. Conversely, identification of adenovirus in a patient specimen by PCR, followed by further characterization of the amplicon, allows for the identification of the virus at the species or type level more expeditiously. For example, typing of enteric adenovirus by PCR by using enteric adenovirus-specific primers and restriction enzyme analysis has been described (3, 4). Likewise, a method for determining oculopathogenic serotypes of species D by a PCR assay has been reported (1). Multiplex PCR-based assays have been developed for detection and identification by amplicon size of selected serotypes or species (22, 30, 31). Finally, digestion by several different restriction enzymes of PCR-derived amplicons has been proposed to determine the species or serotype of an isolate (5, 21).

Our approach is closest to a report describing a PCR assay targeting a region in the hexon gene encompassing all seven hypervariable regions (HVRs), followed by sequencing the amplicon (35). However, this method was perceived to be suboptimal for the clinical laboratory, since it entails nested long PCR, followed by six sequencing reactions, and was further hampered by the incompleteness of the sequence database for all adenovirus serotypes. Accordingly, we designed a PCR assay with a single primer pair targeting conserved segments that bracketed the HVR-7 of the hexon gene (13). This assay can detect all known serotypes of human adenoviruses with high sensitivity, and sequencing of the amplicon enables serotype determination. To complete the sequence database for all adenovirus serotypes, amplicons containing the sequence of the

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TABLE 1. Adenovirus strains used in this study[a]

Serotype	Strain origin	Amplicon size (bp)	GenBank accession no.
1	Laboratory collection	617*	X67709*
2	Laboratory collection	629*	J01917*
3	Laboratory collection	614*	X76549*
4	Laboratory collection	620*	X84646*
5	Laboratory collection	617*	J01966*
6	ATCC VR-1083	623*	X67710*
7	Laboratory collection	605*	X76551*
8	Laboratory collection	620*	X74663*
9	ATCC VR-1086	626*	X74657*
10	ATCC VR-1087	626†	AY288105†
11	ATCC VR-12	614*	AB018424*
12	Laboratory collection	614*	X73487*
13	ATCC VR-1090	629†	AY288106†
14	ATCC VR-1091	608*	AB018425*
15	ATCC VR-1092	626†	AY288107†
16	ATCC VR-1093	632*	X74662*
17	ATCC VR-1094	626†	AY288108†
18	Laboratory collection, ATCC VR-1095	605†	AY288109†
19	ATCC VR-1096	626*	X98359*
20	ATCC VR-1097	626†	AY288110†
21	ATCC VR-1098	611†	AY288111†
22	ATCC VR-1100	611†	AY288112†
23	ATCC VR-1101	614†	AY288113†
24	ATCC VR 1102	608†	AY288114†
25	ATCC VR-223	626†	AY288115†
26	ATCC VR-1104	626†	AY288116†
27	ATCC VR-1105	623†	AY288117†
28	ATCC VR-1106	623†	AY288118†
29	ATCC VR-1107	626†	AY288119†
30	ATCC VR-273	629†	AY288120†
31	Laboratory collection	608*	X74661*
32	ATCC VR-625	626†	AY288121†
33	ATCC VR-626	626†	AY288122†
34	ATCC VR-716	611*	AB018426*
35	Laboratory collection	614*	AB018427*
36	ATCC VR-913	617†	AY288123†
37	ATCC VR-929	629*	X98360*
38	ATCC VR-988	620†	AY288124†
39	ATCC VR-932	608†	AY288125†
40	Laboratory collection	611*	L19443*
41	Laboratory collection	608*	X51783*
42	ATCC VR-1304	626†	AY288126†
43	ATCC VR-1305	608†	AY288127†
44	ATCC VR-1306	614†	AY288128†
45	ATCC VR-1307	626†	AY288129†
46	ATCC VR-1308	608†	AY288130†
47	ATCC VR-1309	623†	AY288131†
48	ATCC VR-1406	614*	V20821*
49	ATCC VR-1407	623†	AY288132†

[a] Symbols: *, the amplicon size was deduced from the sequence built from the primer sequences and the hexon gene sequence whose accession number is listed; †, the amplicon sequence was determined in this study (the sequence internal to the primers was deposited in GenBank under the given accession number).

HVR-7 that had not been previously documented were sequenced. Finally, as a demonstration of principle, the clinical usefulness of this method was demonstrated on a small number of representative clinical specimens.

MATERIALS AND METHODS

Viral strains. Adenovirus strains for each serotype used in the present study, described in Table 1, were either purchased from the American Type Culture Collection (ATCC) or were laboratory isolates typed by virus neutralization by standard methods (10). Only ATCC strains were used for new sequence determinations.

Isolation in cell culture. Viral strains were amplified in either 293 cells (23) or HeLa cells, as described previously (10, 11). Viral stocks, consisting of cell lysates, were kept at −80°C. For quantitative determination of the PCR sensitivity, virus stocks were titrated in infectious units/milliliter, as described previously (9). Conversion into genome copies was performed by using established ratios for each serotype (11, 24).

DNA extraction. Extraction of DNA from viral stocks (cell lysates), urine, nasopharyngeal swabs and eye swabs was performed by using a guanidinium thiocyanate-based method as described previously (28). For sequencing amplicons of reference strains, DNA was extracted directly from an aliquot taken from the ATCC vial in order to minimize the possibility of cross-contamination. DNA was extracted from blood and pleural fluid specimens by using the blood QIAAmp extraction kit (Qiagen) according to the manufacturer's recommendations. DNA was extracted from stool specimens by mixing them with an equal volume of phosphate-buffered saline. After centrifugation, DNA was extracted from the supernatant fraction by the guanidinium thiocyanate method outlined above.

Primers. Primers were designed to target conserved segments bracketing the HVR-7 of the hexon gene (13). The sense primer AD1 has the sequence 5′-CTG ATG TAC TAC AAC AGC ACT GGC AAC ATG GG-3′. The antisense primer AD2 has the sequence 5′-GCG TTG CGG TGG TGG TTA AAT GGG TTT ACG TTG TCC AT-3′.

PCR. Each reaction was done in a 0.6-ml tube (Diamed PRE 050) in a total volume of 50 μl overlaid with 50 μl of mineral oil. Each reaction contained 5 μl of 10× Cetus buffer II (Perkin-Elmer), 5 μl of 25 mM $MgCl_2$, 5 μl of deoxynucleoside triphosphate mix (Pharmacia; the concentration of each deoxynucleoside triphosphate was 2 mM), 25 pmol of each primer, 0.5 μl (2.5 U) of Ampli*Taq* Gold (Perkin-Elmer), and molecular-grade double-distilled water to a volume of 40 μl. All components, except the templates, were prepared as a master mix, which was then divided into aliquots in separate tubes, to which 10 μl of template DNA, dissolved in double-distilled water, was added. PCR was done by using a Stratagene Robocycler 40. The cycling parameters consisted of an initial denaturing step of 10 min at 95°C, followed by 37 cycles consisting of denaturation at 95°C for 1 min, annealing at 51°C for 1 min, and elongation at 72°C for 1 min, with a final incubation at 72°C for 3 min. After PCR, a 10 μl of each reaction mixture was subjected to electrophoresis on a 1.5% agarose gel containing ethidium bromide. The bands were visualized on a UV transilluminator and photographed. Precautions against PCR contamination, described previously (28), were strictly adhered to.

Sequencing. Both strands of the amplicons were sequenced by automated sequencing with the PCR primers as sequencing primers. Sequencing was done at the DNA Sequencing Facility, Centre for Applied Genomics, Hospital for Sick Children.

Sequence analysis. Sequence editing and analysis were done by using the programs Gene Runner for Windows (ver. 3.05; Hasting Software). Sequence alignments were calculated by using ClustalX for Windows version 1.81 (36), with the default parameters for gap opening and gap extension. Phylogenetic trees were inferred by using TREECON for Windows version 1.3.b (37), by using a distance method. In brief, the distance was calculated without correction, taking the gaps into account; the tree topology was inferred by the neighbor-joining method, and the tree was rerooted at the internode. Bootstrap analysis was done with 1,000 replicates.

RESULTS

Primer design. The aim of the present study was to achieve a PCR assay for adenoviruses that would amplify all known serotypes of adenoviruses with a single primer pair and that would permit serotype determination by sequencing the amplicon. At the outset of the study, the complete sequence of the hexon gene was available for some serotypes, including at least one serotype of each species. An alignment of the hexon gene sequences was computed by using sequences from representative serotypes of species A (serotypes 12 and 31), B (serotypes 3, 7, 11, 14, 16, 34, and 35), C (serotypes 1, 2, 5, and 6), D (serotypes 8, 9, 19, 37, and 48), E (serotype 4), and F (serotypes 40 and 41) (for GenBank accession numbers, see Table 1). This alignment revealed two suitable highly conserved regions

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against which primers could be targeted. The primers selected (see Materials and Methods) bracketed a region of 605 to 629 nucleotides whose sequence was highly variable between serotypes. Comparison of this region with previously reported alignment of hexon protein sequences (13) revealed that these primers bracketed the HVR-7 of the hexon gene. Furthermore, it has been established that the HVR-7 contains neutralizing epitopes (13), and therefore its sequence would very likely be correlated with the serotype.

Detection of adenoviruses by PCR and sensitivity determination. DNA extracted from adenoviruses serotypes 1 to 49 from the strains listed in Table 1 was subjected to PCR. An amplicon of the expected size was readily obtained for each serotype. The sensitivity of the assay was determined by performing PCR on 10-fold serial dilutions of the DNA extracted from representative serotypes of each species whose infectious titers had been determined. The number of genome copies detected was assumed to be equivalent to the number of viral particles, which in turn was estimated by using established ratios of infectious units to particle numbers for each serotype (11, 24). The measured sensitivities were 15 genome copies for serotype 18 (species A), 10 genome copies for serotype 10 (species B), 200 genome copies for serotype 5 (species C), 9 genome copies for serotype 13 (species D), 36 genome copies for serotype 4 (species E), and 4 genome copies for serotype 40 (species F).

PCR specificity. The identity of amplicons was verified by sequencing. In addition, amplicons of the size expected for adenoviruses were not observed when the PCR assay was tested on template DNA from herpes simplex 1, Epstein-Barr virus, cytomegalovirus, polyomavirus BK, parvovirus B-19, *Bordetella pertussis*, *Mycoplasma pneumoniae*, or human DNA from blood and tissue samples.

Sequencing and phylogenetic analysis. Figure 1 displays a phylogenetic tree built from an alignment of the amino acid sequences of the amplicons of each serotype prototype. Figure 2 displays a phylogenetic tree built from an alignment of the nucleotide sequences of the amplicons of each serotype prototype and of illustrative clinical isolates. The sequences were deduced from the primer sequences and the HVR-7 sequence in between. For the serotypes used in the primer design, we used the GenBank sequence of the hexon gene (Table 1). For all of the other serotypes, amplicons obtained from ATCC strains were sequenced directly. To eliminate any deviations from growth of these viruses in culture, an aliquot taken directly from the ATCC vial was used for extraction of DNA as a template for PCR. The sequences internal to the primers were deposited in GenBank under the accession numbers given in Table 1. For all of the amplicon sequences determined in the present study, a BLAST analysis in the GenBank database was done. A 100% agreement was obtained with existing GenBank sequences for serotypes 10, 17, 21, 22, 24, 46, and 47; an agreement of 99% was obtained for serotypes 23, 26, and 45; and an agreement of 98% was obtained for serotype 15. Furthermore, our sequences extended the available partial sequence of that region of the hexon gene for serotypes 10, 22, 24, 26, 45, 46, and 47. Lastly, our sequences constituted new, previously unavailable information for serotypes 13, 18, 20, 25, 27, 28, 29, 30, 32, 33, 36, 38, 39, 42, 43, 44, and 49.

As shown in Fig. 1 and 2, each serotype is clearly distinct as shown by phylogenetic analysis. Serotypes belonging to the same species cluster together. It is noteworthy to observe that serotypes 11, 14, 34, and 35, which encompass the species B2, cluster together within the species B in the phylogenetic tree built from the nucleotide sequences alignment (Fig. 2).

Serotype determination by PCR and/or sequencing from clinical samples and from serotype candidates 50 and 51. As a demonstration of the clinical utility of this process of PCR followed by sequence determination, detection and serotype determination were performed on a limited number of illustrative clinical specimens. The criterion for serotype assignation was that of the shortest distance from a prototype strain, as deduced from the phylogenetic tree (Fig. 2).

Specimens W21 and H28 were eye swabs from two children who presented with uncomplicated conjunctivitis. Testing by PCR yielded a positive result and the phylogenetic analysis of the amplicon determined that these specimens contained adenovirus serotype 3 (Fig. 2). Adenoviruses were also isolated in cell culture.

Specimen M41 was a nasopharyngeal swab from a patient with an upper respiratory infection. Testing by PCR and sequencing of the amplicon determined that this was an adenovirus serotype 3 (Fig. 2). Adenovirus was also identified by immunofluorescence microscopy.

Specimens W16 and X66 were stools in which adenoviruses were demonstrated by electron microscopy. Testing by PCR and sequencing of the amplicon determined that these were both adenovirus serotype 41 (Fig. 2).

The sequence WB01 was identical in the three amplicons obtained from the blood, pleural fluid, and stool samples of a bone marrow transplant recipient with sepsis. No other infectious agent could be demonstrated in this patient suffering from a disseminated adenovirus infection. This isolate clearly belonged to serotype 2 (Fig. 2).

Specimens N52 and N55 were urine samples from two bone marrow transplant recipients who developed hemorrhagic cystitis. Adenoviruses along with polyomaviruses, were visualized in one specimen by electron microscopy. Both specimens were positive by the adenovirus PCR and, after sequencing of the amplicons, were shown to contain serotypes 35 and 34, respectively (Fig. 2).

Lastly, strains VR1501 and VR1502, candidate prototypes for serotypes 50 and 51, respectively, were obtained from the ATCC. In the PCR assay, an amplicon was detected from the DNA of both strains. Phylogenetic analysis of the sequences of these amplicons supports their status as distinct serotypes since the distances between these isolates and the closest prototype strains were considerably greater than for clinical isolates and, in fact, comparable in magnitude to the distances separating prototype strains. As illustrated in Fig. 2 VR1501 would be classified into species D and VR1502 into species B1. For these two strains, the sequence internal to the primers was deposited in GenBank (accession numbers AY303952 and AY303953).

DISCUSSION

A PCR assay, applicable to clinical specimens, was developed that could detect all known serotypes of human adenovirus with high sensitivity and allow for serotype determination by sequencing of the amplicons. The primer pair selected is

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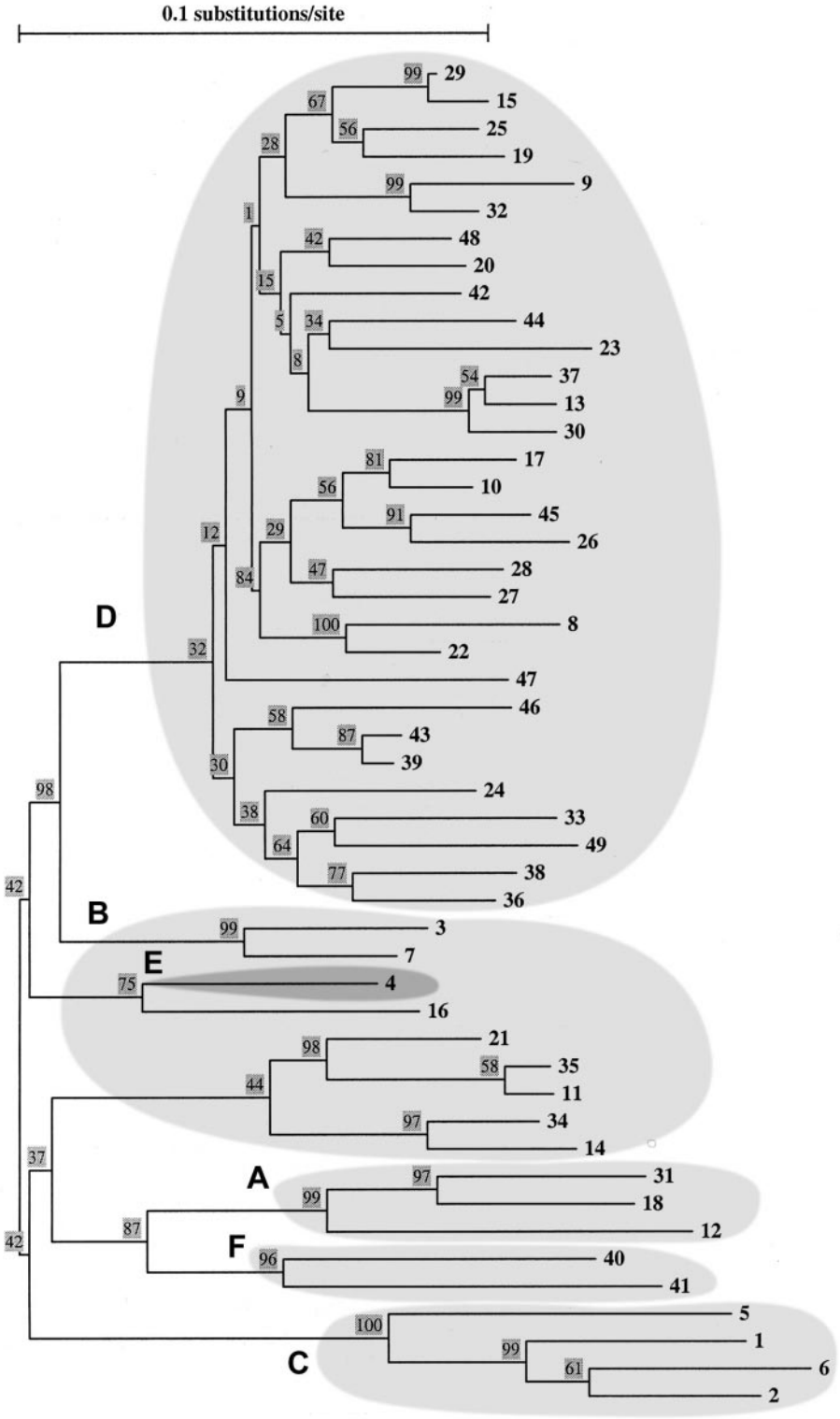

FIG. 1. A phylogenetic tree from the amino acid sequences of the amplicons of the prototype strains was inferred from an alignment calculated with ClustalX for Windows version 1.81; the tree was built with the program TREECON for Windows version 1.3.b by the neighbor-joining method, as described in the text. Prototype strains are identified by the serotype number at the end of the branches. Serotypes belonging to the same species cluster together; the species are labeled A to F. Bootstrap values (from 1,000 replicates) are expressed as a percentage for each node.

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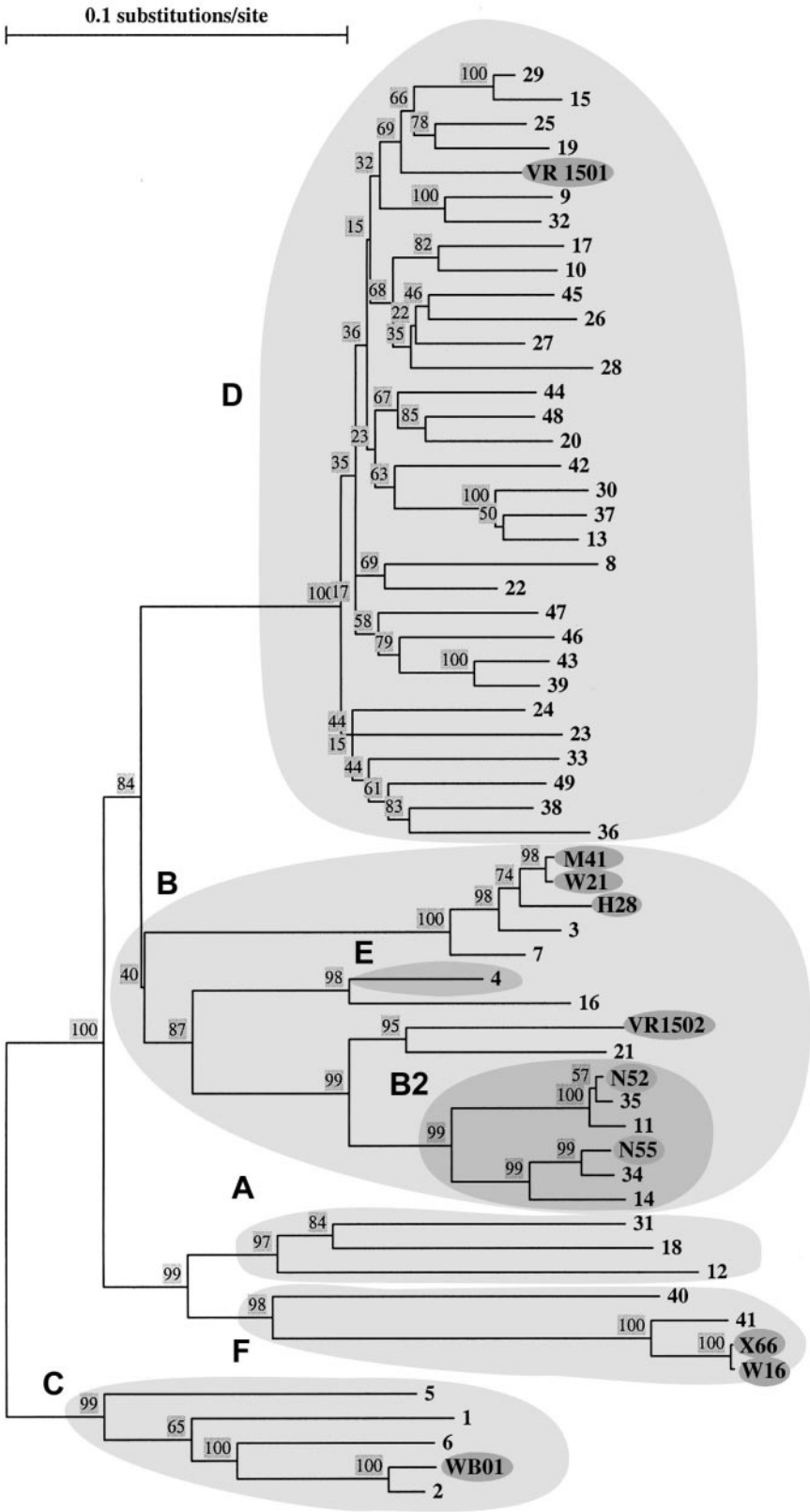

FIG. 2. A phylogenetic tree from the nucleotide sequences of the amplicons was inferred from an alignment calculated with ClustalX for Windows version 1.81; the tree was built with the program TREECON for Windows version 1.3.b by the neighbor-joining method, as described in the text. Prototype strains are identified by the serotype number at the end of the branches; clinical isolates and strains VR1501 and VR1502 (candidate serotypes 50 and 51) identifiers are highlighted at the end of their branches. Serotypes belonging to the same species cluster together; the species are labeled A to F, with the subspecies B2 also illustrated. Bootstrap values (from 1,000 replicates) are expressed as a percentage for each node.

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based on conserved sequences bracketing the HVR-7 of the hexon gene. Depending on the species, our sensitivity was estimated at between 200 and 4 genome copies; the latter value was obtained for the species F and, given the high ratio of viral particles to infectious units for that species (11) compared to the others, this measurement is arguably the least certain. Even so, based on other species, the sensitivity remains between 200 and 9 genome copies. Using newly derived sequences of the amplicons and those already deposited in GenBank, phylogenetic trees were constructed that showed that the amplicon of each serotype had a readily identifiable sequence.

It is noteworthy that, based on this sequence analysis, the serotypes cluster according to the species into which they are classified, except perhaps for species E (serotype 4), which localizes within species B (Fig. 1 and 2). It is known that the E1, E2, and hexon gene sequence of adenovirus 4 are similar to that of species B, whereas the penton gene sequence is similar to that of species C, and it is speculated that adenovirus 4 arose in part through recombination (33). Based on this and other properties, such as the G+C content and hemagglutination, adenovirus 4 is still considered the sole serotype of a distinct species (7). It is also relevant to note that within species B, serotypes 11, 14, 34, and 35, which comprise species B2 (15, 32), form a cluster in the phylogenetic tree built from the nucleotide sequences. Adenoviruses belonging to species B2 have been shown to differ from those of subgroup B1 in their use of cellular receptors and their tissue tropism (32). The bootstrap values (Fig. 1 and 2) are overall quite high, and especially at the nodes between species; the values are, however, less good for some nodes within species D. However, the goal of the present study is not the establishment of the phylogeny of adenoviruses, or even the precise measurement of the genetic distance between serotypes: such calculations ought to be done with much larger segments of the genome (and ideally complete genome sequences). Rather, since the HVR-7 contains some of the neutralization epitopes on which serotyping is based (13), its sequence ought to correlate with the serotype. The phylogenetic trees support this contention, since all serotypes are quite distinct. The assignment of a serotype to a clinical isolate is done through calculating the shortest distance between the isolate and a prototype (visual inspection is sufficient for that purpose). This is more conveniently done by using the nucleotide sequence, since this approach precludes the additional step of translating the sequence. It should be noted, however, that our typing scheme, like other typing methods, could fail to recognize recombinant strains, and this should be kept in mind when the results are interpreted. Recombination between adenoviruses has been well documented in vitro and especially between strains belonging to the same species (33). In a recent survey 2,301 strains collected between 1973 and 1992 were typed by De Jong et al. (15); all but 9 isolates could be typed, which suggests that recombinant strains in the field may occur relatively infrequently.

The clinical relevance of our approach was illustrated by its applicability to a limited number of representative clinical specimens. In all cases, the adenovirus serotype identified was consistent with a clinical presentation in which it had been previously described (27, 34). Furthermore, these examples illustrate several situations where the knowledge of the serotype has proven valuable for patient management. Thus, in ocular infections, it was desirable to distinguish between serotypes associated with simple conjunctivitis (such as serotype 3) and serotypes associated with the more severe epidemic keratoconjunctivitis (associated mainly with serotypes 8, 19, and 37). Epidemic keratoconjunctivitis raises issues of infection control, is associated with significant morbidity (1, 26, 27, 35), and may be amenable to therapy with topical cidofovir (26). Identification of the adenovirus serotype is of major concern in immunocompromised patients. In this regard, adenovirus serotypes 40 and 41, the so-called enteric adenoviruses, are a common cause of gastroenteritis in children (27, 34) and account for ca. 55% of the adenoviruses identified in the gastrointestinal tract (10); however, they have not been recovered in systemic disseminated infection in immunodeficient patients. In contrast, adenoviruses 1, 2, 5, and 6, which can also be recovered from the gastrointestinal tract, have been documented to cause disseminated infections in immunodeficient patients, and their recovery from stools, in this context, may prompt investigation for adenovirus disseminated disease. Indeed, in a patient described in the present study, adenovirus 2 was detected by PCR in stools, blood, and pleural fluid. This bone marrow transplant recipient presented at the time with sepsis for which no other agent could be identified. Lastly, the detection and typing of adenoviruses from two bone marrow transplant recipients with hemorrhagic cystitis had important management implications. These two patients presented with hemorrhagic cystitis within a 1-week period on the same ward, raising the issue of a common source and nosocomial transmission. The prompt identification of the viruses as serotypes 34 and 35, both of which have been associated with urinary tract infection (27, 34), provided definitive evidence that these were two separate events and therefore probably not due to infection from a common source.

Amplicons were successfully obtained from ATCC strains VR1501 and VR1502, candidate strains for serotypes 50 and 51, respectively (15), and their sequences were compared by phylogenetic analysis with those of the existing 49 serotypes (Fig. 2). The sequences from these two strains are clearly distinct from those of other serotypes and much more distant from prototypes than the clinical isolates. Our findings therefore support the proposal that these isolates represent new serotypes (15). However, our findings are inconsistent with this proposal in terms of species designation. Based on conventional findings, serotype 50 has characteristics of species B1, and serotype 51 has characteristics of species D (15), whereas our sequence analysis indicates the converse species association. This raises the intriguing possibility that these new serotypes may have arisen, at least in part, through recombination, and further sequence data from the hexon gene will be required to clarify this point. Alternatively, the serotypes may have been inadvertently mislabeled between the time of initial isolation and their receipt in our laboratory. We were very careful in our approach to ensure that a portion of each stock received from ATCC was faithfully analyzed by extracting the DNA directly from the ATCC vial and not after further passaging in cell culture. When the above discrepancy was noted, the entire process was repeated on individual ATCC stocks to eliminate the possibility of clerical error. Pending a resolution of this inconsistency, the sequence of these amplicons was

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reported to GenBank under the ATCC number rather than the designated serotype number.

In summary, we developed a simple PCR assay that uses a single primer pair for the rapid, sensitive detection of all known human adenoviruses and permits accurate typing by sequencing of the amplicons. With our deposit in GenBank of the amplicon sequences of all serotypes not yet reported, the database needed to provide unambiguous serotype determination is now complete. The assay has proven useful in a clinical setting and is expected to contribute to more rapid diagnosis of severe adenovirus-associated diseases, as well as to further our understanding of the epidemiology of these agents.

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