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Outbreak of Acute Respiratory Infection among Infants in Lisbon, Portugal, Caused by Human Adenovirus Serotype 3 and a New 7/3 Recombinant Strain▽

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Human adenoviruses (AdVs) typically cause mild illnesses in otherwise healthy hosts. We investigated a pediatric outbreak of acute respiratory infection with fatal outcomes that occurred in Lisbon, Portugal, in 2004. Biological specimens were collected from 83 children attending two nurseries, a kinesiotherapy clinic, and the household of a nanny. Adenovirus infection was confirmed in 48 children by PCR and virus isolation. Most (96%) isolates were classified as being of subspecies B1. Phylogenetic analysis of fiber and hexon gene sequences revealed that most infants were infected with AdV serotype 3 (AdV3) strains. Infants attending one nursery harbored a new recombinant strain containing an AdV serotype 7 hexon and serotype 3 fiber (AdV7/3). Both the AdV3 and the AdV7/3 strains caused fatal infections. Two different serotype 3 strains were circulating in Lisbon in 2004, and the new AdV7/3 recombinant type originated from only one of those strains. These results demonstrate that recombination leads to the emergence of new adenovirus strains with epidemic and lethal potential.

Human adenoviruses (HAdVs) have been associated with a wide spectrum of clinical diseases with respiratory and gastrointestinal symptoms (23, 32). Severe illness can occur in newborns, elderly individuals, and patients with underlying medical conditions. In otherwise healthy adults, infections caused by human adenoviruses do not represent a life-threatening clinical condition. Adenoviruses are characterized by a linear double-stranded DNA genome of 2 to 45 kbp that encodes 30 to 40 proteins (6). HAdV comprises 51 serotypes (HAdV-1 to HAdV-51), on the basis of type-specific antiserum-mediated neutralization of infectivity (10). The serotypes can be divided into seven species, named HAdV-A to HAdV-G, on the basis of hemagglutination inhibition and biochemical criteria (13). HAdV-B is further classified into subspecies B1 and B2, which use different cellular receptors for viral entry (29). These variants can be segregated by different geographic areas, time periods, and clinical conditions.

Serotype identification is critical for epidemiological surveillance, the detection of new strains, assessment of treatment efficacy, and understanding the pathogenesis of HAdV. For example, acute respiratory disease is primarily caused by HAdV-B1 serotypes 3, 7, 16, and 21; HAdV-B2 serotypes 11 and 14; and HAdV-E serotype 4 (8, 23, 25, 28, 35, 38, 41). Respiratory infections caused by HAdV-B1 serotypes 3 and 7 (16) and HAdV-B2 serotype 14 (17) are potentially fatal. Neutralization tests are the classical reference method used for the typing of adenovirus and require virus isolation from infected organs or tissues (20). The main type-specific neutralizing epitope, the ε determinant, consists of loop 1 (L1) and loop 2 (L2) on the hexon protein, the major capsid protein and the most abundant structural protein (26). Cases of the failure of neutralization with the available antisera require extensive cross-neutralization studies to define a new HAdV type. To circumvent the practical problems associated with traditional serum neutralization studies, molecular methods for the typing of adenovirus have been established. Examples are restriction fragment length polymorphism (RFLP) analysis of adenoviral DNA (16), PCR-based assays (1, 36), and microarray-based methods (36). However, these methods cannot discriminate between all serotypes and do not allow detailed studies of molecular epidemiology and viral evolution to be performed.

More recently, analysis of the nucleotide and amino acid sequences from different genes has shown that adenovirus species form three distinct phylogenetic clusters: HAdV-C belongs to cluster 1; HAdV-A and HAdV-F belong to cluster 2; and HAdV-B, HAdV-D, and HAdV-E belong to cluster 3 (6, 22). In addition, phylogenetic analysis of selected gene fragments has increasingly been used to classify human adenoviruses at the serotype and species levels (7, 19, 40), to detect cases of coinfection with multiple adenoviral species (36, 38), and to identify new recombinant strains formed between similar species (18, 37, 41) or different species (19). Finally, phy-

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TABLE 1. Epidemiological, clinical, and virological characteristics of HAdV-B-infected infants

Patient	Age (mo)	Symptom severity[a]	Place of infection	Serotype		
				Hexon gene		Fiber gene, phylogenetic analysis
				PCR and REA[b]	Phylogenetic analysis	
PA2	14	++	Kindergarten A	7	7	3
PA3	22	+	Kindergarten A	7	ND[c]	ND
PA4	17	+	Kindergarten A	7	ND	ND
PA5	23	+	Kindergarten A	7	ND	ND
PA6	21	+	Kindergarten A	7	7	ND
PA17	27	+++	Kindergarten A	7	7/7[d]	3
PA23	18	+++	Kindergarten A	7	ND	ND
PA27	6	++++ (D)	Kindergarten A	7	7/7[d]	3
PA57	23	++	Kindergarten A	7	7	3
PA28	6	++	Kindergarten A	7	ND	ND
PA19	NA[e]	0	Nanny	3	ND	ND
PA22	20	++++ (D)	Nanny	3	3/3/3/3/3/3/3[d]	3/3/3/3/3/3/3[d]
PA25	6	++	Kindergarten B	3	ND	ND
PA32	15	0	Kindergarten B	3	3	3
PA33	21	0	Kindergarten B	3	ND	ND
PA35	32	0	Kindergarten B	3	ND	ND
PA37	33	0	Kindergarten B	3	ND	ND
PA38	53	0	Kindergarten B	3	3	3
PA39	11	+++	Kindergarten B	3	ND	ND
PA40	32	+++	Kindergarten B	3	ND	ND
PA41	23	+++	Kindergarten B	3	3	ND
PA42	13	+++	Kindergarten B	3	3/3[d]	3
PA64	7	+	Kindergarten B	3	3	3
PA65	22	++	Kindergarten B	3	ND	ND
PA77	13	0	Kindergarten B	3	ND	ND
PA80	6	0	Kindergarten B	3	ND	ND
PA81	4	+++	Kindergarten B	3	ND	ND
PA82	5	++	Kindergarten B	3	ND	ND
PA26	13	+++	Kinesiotherapy clinic	3	3	3
PA29	11	+++	Kinesiotherapy clinic	3	3	3
PA30	5	+++	Kinesiotherapy clinic	3	3	3
PA31	3	++	Kinesiotherapy clinic	3	3	3
PA44	1	0	Kinesiotherapy clinic	3	ND	ND
PA46	8	0	Kinesiotherapy clinic	3	ND	ND
PA50	16	0	Kinesiotherapy clinic	3	ND	ND
PA54	1	+++	Kinesiotherapy clinic	3	ND	ND
PA55	4	++	Kinesiotherapy clinic	3	ND	ND
PA56	0	+	Kinesiotherapy clinic	3	ND	ND
PA58	14	++	Kinesiotherapy clinic	3	ND	ND
PA60	9	0	Kinesiotherapy clinic	3	ND	ND
PA61	35	++	Kinesiotherapy clinic	3	ND	ND

[a] 0, no symptoms; +, mild symptoms; ++, moderately severe symptoms; +++, severe symptoms; ++++ (D), death.
[b] Results obtained using a general PCR combined with restriction endonuclease analysis.
[c] ND, not done.
[d] Results based on sequences from isolates obtained from different tissues.
[e] NA, data not available.

logenetic analysis has become an important tool in the epidemiological investigation of many disease outbreaks caused by adenovirus (11, 17, 27, 40, 41). In the present study, we have used epidemiological, virological, and molecular phylogenetic methods to investigate the causes and origin of a recent outbreak of acute respiratory infection in Lisbon, Portugal, that resulted in the deaths of two children.

MATERIALS AND METHODS

Patients and specimens. Biological specimens were collected during an outbreak of respiratory infection that occurred in Lisbon in 2004 and that mainly affected children (median age, 17 months; maximum age, 117 months; minimum age, 0 months) (Table 1). The alert came with the hospitalization of five children with viral pneumonia with suspicion of an adenoviral etiology; and two of those children, one female and one male 6 and 20 months of age, respectively, died.

These cases led to an epidemiological investigation focused on infants attending (i) the nursery of one of the deceased infants (patient PA27, kindergarten A), (ii) the home of the nanny of the other deceased infant (patient PA22), and (iii) a respiratory kinesiotherapy clinic attended by the remaining three children. Later, other biological specimens were collected from children from a second nursery (kindergarten B) who were also hospitalized. Samples collected from symptomatic children attending a third nursery (kindergarten C) were used as controls. During this epidemiological investigation, a total of 128 biological specimens were collected from 83 children. More precisely, at least one sample from the respiratory tract (a respiratory secretion and/or nasopharyngeal swab specimen) and a fecal sample from each child were collected and analyzed. *Postmortem* biopsy specimens of the lungs and liver from one of the deceased children were also studied. All biological specimens (respiratory and fecal sam-

ples) were analyzed by PCR, and those positive for adenovirus by PCR (48 specimens) were submitted for virus isolation. Because of the limited amount of reference serum available, only a subset of 17 samples was evaluated by a microneutralization assay. These samples were from the 2 deceased children, 3 cases who presented with more severe symptoms and who were close contacts of kindergarten A, and 12 selected cases from kindergarten B and from the kinesiotherapy clinic. Forty-one species B-positive specimens were further characterized genetically by PCR-restriction enzyme analysis (REA) of the hexon gene. The hexon and fiber genes of the isolates from 15 patients who presented with higher viral loads and for whom the amount of the original specimen was not a limiting factor were sequenced. Informed consent to participate in the study was obtained from the parents of the children. The study was reviewed and approved by the ethics committees of the participating institutions.

Extraction of nucleic acids, PCR amplification, and sequencing. Viral DNA was extracted from the clinical specimens by a guanidine thiocyanate-silica binding method adapted from the method of Boom et al. 1990 (3) and was stored at −80°C. On arrival, all specimens were tested for adenovirus infection by an adenovirus-specific nested PCR (2). All adenoviruses were further subtyped by multiplex PCR with primers targeting the fiber gene (39). The fiber gene nucleotide sequences were obtained by a cycle sequencing reaction with the PCR primers and a BigDye Terminator kit (Applied Biosystems).

Genetic analysis of the hexon gene was subsequently performed for all species B-positive samples. PCR-REA was carried out by use of an adaptation of the protocol described by Allard et al. (1), which used 20 μl of viral DNA and 80 μl of a PCR mixture containing 0.5 μM each primer, 0.2 μM deoxynucleoside triphosphates, 3 mM $MgCl_2$, 1 U of *Taq* polymerase, and 10 μl of 10× PCR buffer. Enzymatic digestion was carried out with three enzymes (AcsI, Bsp1286I, and HinfI) and 10 μl of the PCR product under the conditions specified by the manufacturer. The restriction enzyme digests were loaded onto a 3% agarose gel, and gel electrophoresis was run for 3 h at 75 V in TBE (Tris-borate-EDTA) buffer. After staining of the gel with ethidium bromide (1 μg/ml), the DNA fragments were photographed under UV light. For sequencing analysis, part of the hexon gene was amplified by PCR with the primers and under the conditions described previously (4, 15, 24). Sequencing was performed with the PCR primers and a BigDye Terminator kit (Applied Biosystems).

Virus isolation and neutralization assay. All biological specimens with an adenovirus-positive PCR result were inoculated onto HEp-2, Vero, MRC-5, and RD cells for virus isolation. The nasopharyngeal swab specimens were previously treated with 2.0 ml of tryptose supplemented with gelatin and a mix of antibiotics and an antifungal agent. The fecal samples were diluted in phosphate-buffered saline (1/5), the mixture was centrifuged, and the supernatant was treated with chloroform (antibiotics and an antifungal were added). The processed biological specimens were inoculated into tubes with a confluent monolayer of HEp-2, Vero, MRC-5, or RD cells. The tubes were incubated at 37°C and observed daily for a cytopathic effect (CPE; rounded cells). The serotypes of the isolates were determined by a microneutralization assay with 2-fold dilutions of type-specific hyperimmune rabbit antisera (anti-type 3, anti-type 4, and anti-type 7) produced in our laboratory and empirical determination of the virus dosages which induced a CPE on 100% of the HEp-2 or MRC-5 cells after 7 days of incubation at 37°C (5, 20). For serotype determination, the highest dilution which completely inhibited the development of a CPE (100% neutralization) and the highest dilution which inhibited 75% of the CPE (75% neutralization) were considered. The serotype identification was confirmed when the neutralization titer observed was within 16-fold of the titer observed when the same antiserum was tested against the homologous prototype virus. Isolates neutralized by both anti-type 7 antiserum and anti-type 3 antiserum but not by anti-type 4 antiserum were considered to present double cross-reactivity.

Phylogenetic analysis. The nucleotide sequences produced in this study were first compared with all sequences present in GenBank by pairwise alignment by use of the BLAST program. The 10 sequences with the highest alignment scores were recovered. Reference genomic sequences of each adenovirus species within the *Mastadenovirus* genus were also obtained from GenBank. All sequences were aligned by using the CLUSTAL X program; the alignments were manually edited with the GENEDOC program.

For the fiber gene, phylogenetic reconstructions were done with an alignment of 379 nucleotides of 53 taxa (positions 31379 to 31707 in reference strain AY599836.1 of HAdV-B1 serotype 3); an alignment of 257 nucleotides of 60 taxa was performed as used for the hexon gene (positions 18479 to 18734, corresponding to amino acids 22 to 106). Maximum-parsimony and maximum-likelihood analyses with bootstrap analysis (1,000 replicates) were used for inference of the phylogenetic trees. The nucleotide distances were also calculated for the hexon and fiber sequences by using the Kimura two-parameter substitution model with different rates of substitution among sites (the γ distribution). The γ shape parameter α was arbitrarily set equal to 1.0. Phylogenetic reconstructions and nucleotide distances were determined by the use of MEGA (version 4) software (34).

Nucleotide sequence accession numbers. The sequences determined in this study have been assigned GenBank accession numbers GQ922009 to GQ922027 (fiber genes) and GQ922028 to GQ922051 (hexon genes).

RESULTS

Adenovirus infection was confirmed using PCR for 48 children up to 36 months of age. Most of the children were male (62%). Twelve (25%) of 48 infants were from kindergarten A, 20 (42%) were from kindergarten B, 3 (6%) were from the household of the nanny, and 13 (27%) were from the respiratory kinesiotherapy clinic (Table 1). The adenovirus infections were asymptomatic in 17 (35%) infants and caused mild symptoms in 7 (15%) infants, moderate symptoms in 11 (23%) infants, severe symptoms in 11 (23%) infants, and death in 2 (4%) infants. The deaths occurred in one child from kindergarten A and one child attending the household of a nanny (Table 1). By multiplex PCR, 46 (96%) of the positive isolates were classified as HAdV-B, and the remaining 2 were found to belong to species C and D. Virus was isolated from at least one specimen per case studied, resulting in a total of 48 isolates.

Seventeen of the 48 isolates (35%) were subtyped by microneutralization assay. The results revealed that four of the isolates showed double cross-reactivity with AdV serotype 3 (AdV3) and AdV7 antisera. These isolates corresponded to one isolate from a deceased child (patient PA27) and isolates from the three contacts from kindergarten A (patients PA2, PA17, and PA57) (data not shown). Genetic characterization of 41 virus isolates was first done by restriction enzyme analysis of the hexon gene (Table 1). It was found that 31 (76%) infants were infected with serotype 3 viruses and that 10 (24%) were infected with serotype 7 viruses.

Fifteen isolates were subsequently characterized by sequencing and phylogenetic analysis of the fiber gene and/or the hexon gene. Analysis by maximum parsimony revealed that all fiber gene sequences ($n = 14$; 100%) were highly conserved and clustered together within human adenovirus subspecies B1, serotype 3 (Fig. 1A). For the hexon gene, 10 sequences clustered together within HAdV-B1 serotype 3. In addition, five sequences from patients PA2, PA6, PA17, PA27, and PA57 were phylogenetically related to HAdV-B1 serotype 7 virus (Fig. 1B). Similar results were obtained by maximum-likelihood analysis using different models of nucleotide substitution (data not shown). The discordant phylogenies in the hexon and fiber genes of the isolates from these patients indicate that they harbor a recombinant adenovirus strain containing a chimeric capsid composed of a serotype 7 hexon protein and a serotype 3 fiber (AdV7/3). All putative recombinant strains were detected in infants from kindergarten A (Table 1). One of these children (PA27) died of acute respiratory disease. The other fatal infection (PA22) was due to a serotype 3 strain and occurred at a different location (the home of the nanny).

We also determined the nucleotide evolutionary distances and the nucleotide and amino acid similarities between the Portuguese hexon and fiber sequences and compared the

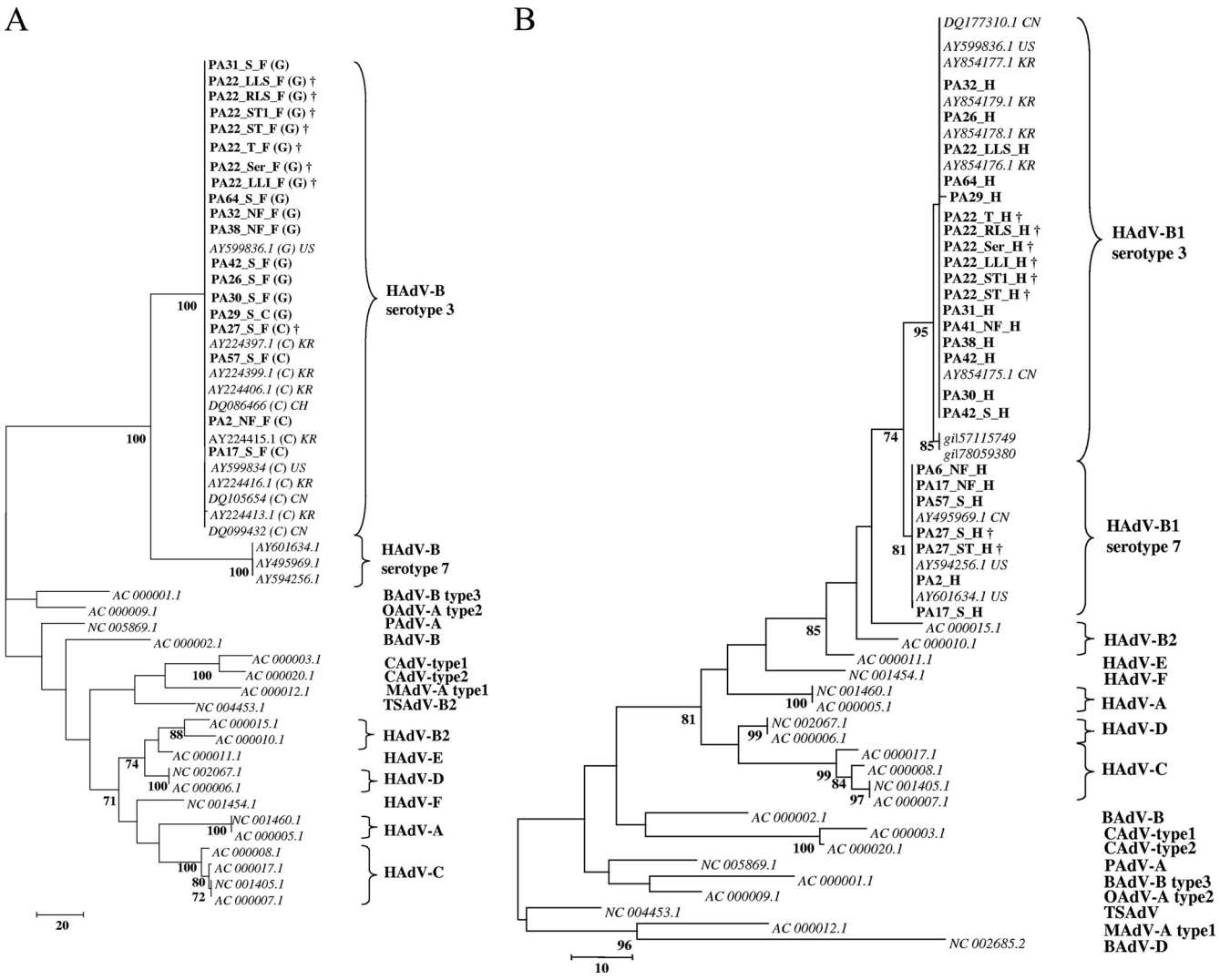

FIG. 1. Evolutionary relationships of adenovirus isolates. The evolutionary history was inferred by using the maximum-parsimony method and the nucleotide sequences of the hexon (B) and fiber (A) genes. The percentages of replicate trees in which the associated taxa clustered together in the bootstrap test (1,000 replicates) are shown below the branches. Only bootstrap values above 70% are shown. The tree is drawn to scale, and the branch lengths were calculated by using the average pathway method and are in units of the number of changes over the whole sequence. Reference *Mastadenovirus* genus strains are indicated in italics and are marked by the GenBank accession number, the name of the host, the country of origin in International Organization for Standardization characters (CN, China; KR, Republic of Korea; US, United States of America; CH, Switzerland), and the serotype number. Species names are abbreviated as follows: BAdV, bovine AdV; OAdV, ovine AdV; PAdV, porcine AdV; CAdV, canine AdV; MAdV, mouse AdV; and TSAdV, tree shrew AdV. S, stool; T and ST, tracheal suction; NF, nasopharyngeal. Postmortem tissue types: LLS, left lung section; RLS, right lung section; LLI, liver lobe. Sequences from Portuguese patients are indicated in boldface. Each patient is designated by PA followed by a number. The *postmortem* tissue used for virus isolation for the deceased patients (patients PA22 and PA27, also designated †) is indicated. The nucleotide at position 93 in the fiber gene (C or G) is indicated in parentheses (see Discussion).

sequences with those of reference strains. The serotype 3 hexon gene sequences from Portugal differed from the homologous reference sequences by 1 to 4 (0.4% to 1.6%) nucleotides. The serotype 7 hexon gene sequences from Portugal differed from the homologous reference sequences at only 1 (0.4%) nucleotide, located at position 244 (an A nucleotide in the reference sequences and a C nucleotide in the Portuguese sequences). This is a nonsynonymous substitution that leads to a serine-to-arginine amino acid replacement at position 82. In contrast, the serotype 7 Portuguese hexon gene sequences differed from the serotype 3 Portuguese hexon gene sequences by 11 (4.3%) residues, all of which were of a synonymous nature (no amino acid replacements occurred). Regarding the nucleotide distances, the hexon genes of serotype 3 strains were also more divergent than those of the serotype 7 strains (Table 2). As was the case for the reference sequences, the fiber gene sequences from the serotype 3 Portuguese isolates were highly homologous (Table 3). In fact, only one nucleotide at position 93 was variable (synonymous substitution) in more than one sequence. In all AdV7/3 recombinant strains, the nucleotide at this position was a C (a TTC serine codon),

TABLE 2. Nucleotide distances between hexon sequences from Portuguese patients and reference isolates[a]

Sequence	No. of patients	Within-group:		Between-group:	
		Genetic distance	SE	Genetic distance	SE
HAdV3 ref.	12	0.003	0.002	0.002	0.001
HAdV3 PT	11	0.001	0.001		
HAdV7 ref.	3	0.000	0.000	0.000	0.000
HAdV7 PT	4	0.000	0.000		

[a] SE, standard error calculated by bootstrap resampling; PT, Portuguese sequences (this study); ref., reference sequences (from GenBank).

whereas in the serotype 3 strains it was a G (a TTG serine codon) (Fig. 1A).

DISCUSSION

In this report we describe epidemiological, virological, and molecular phylogenetic investigations undertaken to identify the cause and the origin of an outbreak of acute and fatal respiratory infection that occurred in Lisbon and that involved 48 infants (median age, 17 months).

Using neutralization, restriction enzyme analysis, and/or phylogenetic analysis of hexon and fiber gene fragments, we were able to accurately identify serotype 3 of the HAdV-B1 subspecies to be the main causative agent of this outbreak. Our results confirm and extend previous findings showing that Ad3 and Ad7 are highly virulent and potentially lethal subtypes, especially for children (8, 12, 16, 25, 38). Sequencing and phylogenetic analysis indicated that infants from one kindergarten were infected with a new recombinant adenovirus strain composed of a serotype 7 hexon and a serotype 3 fiber. As with Adv3, the Adv7/3 recombinant strain caused several severe infections, including one that was fatal. These results demonstrate that the new AdV7/3 recombinant strain is transmissible, epidemic, and highly virulent.

Of note, we could not identify the new AdV7/3 recombinant strain using standard neutralization analysis due to extensive cross-reactivity with reference anti-type 3 and anti-type 7 sera. The type-determining neutralizing epitopes are primarily located in hexon loop 1 and, to a much lesser degree, loop 2 (4, 9). The results therefore suggest that the AdV7/3 strain has a chimeric hexon protein that comprises the type-determining neutralizing epitopes from the original serotype 7 and serotype 3 strains. Sequencing of the entire hexon gene of the recombinant strains will need to be done to investigate this possibility. Nonetheless, our results confirm the limitations of the use of neutralization-based assays for the serotyping of chimeric adenovirus strains (9).

TABLE 3. Nucleotide distances between fiber sequences from Portuguese patients and reference isolates[a]

Sequence	No. of patients	Within-group:		Between-group:	
		Genetic distance	SE	Genetic distance	SE
HAdV3 ref.	11	0.002	0.001	0.004	0.004
HAdV3 PT	13	0.002	0.002		
HAdV7 ref.	3	0.000	0.000	NA	NA

[a] SE, standard error calculated by bootstrap resampling; PT, Portuguese sequences (this study); ref., reference sequences (from GenBank); NA, not applicable.

The serotype 7 partial hexon gene sequences from Portugal differed from the homologous reference sequences by 1 amino acid at position 82 (a serine-to-arginine replacement), and the fiber gene sequences of the serotype 3 isolates differed from each other by only 1 nucleotide (at position 93). Remarkably, in all putative AdV7/3 recombinant strains, the nucleotide at this position was C, whereas in the serotype 3 strains it was G. These results confirm the strong genetic relatedness and slow evolution of these two serotypes (14, 19, 30). On the other hand, these results show that it is possible to find efficient molecular epidemiologic markers of the circulating adenovirus strains involved in disease outbreaks.

The presence of the HAdV-B serotype 7/3 recombinant strain in the infected infants may have one of two explanations: coinfection with both serotypes or infection with a new recombinant strain. Coinfection with HAdV-4 and HAdV-7 strains has recently been observed in several patients with acute respiratory disease (36). On the basis of the sequence analysis, we do not favor this hypothesis because none of the patients in this outbreak seemed to be infected with HAdV-B subtype 7. Instead, our results suggest that two different parental subtype 3 strains were circulating in Lisbon in 2004 and that only one of them gave rise to the new recombinant strain with HAdV-B serotype 7. The HAdV-B serotype 7/3 recombinant strain must have been generated in an unidentified index case in the same year and was readily transmitted to and among the infants of kindergarten A.

Phylogenetic discordances between the fiber gene and/or the hexon gene of several human adenoviruses have been documented before, indicating that intra- and interspecies recombination are relatively frequent events in the evolution of HAdV (7, 19). However, to our knowledge, this is the first description in Europe of a HAdV-B serotype 7/3 recombinant strain causing acute lower respiratory tract infection and death. A potentially similar recombinant strain was previously found in South America (1991 to 1994), where it caused 17 of 18 fatal lower respiratory tract infections in children (12). The wider transmission of the HAdV-B serotype 7/3 recombinant strains may have been prevented by its potential to cause death. Clearly, further studies are needed to better understand the epidemiology and natural history of these recombinant adenoviruses.

Recombination may lead to faster rates of molecular evolution of human adenoviruses, may increase viral fitness, may change cell tropism, and, as seen here and elsewhere, may lead to the generation of new and more virulent strains (12, 21, 31, 33). Recombination is also an important issue for adenovirus-based vaccine development. Thus, a better understanding of the impact of recombination in the epidemiology and pathogenesis of human adenoviruses is urgently required.

In conclusion, human adenovirus serotype 3 and a newly formed HAdV-B serotype 7/3 recombinant strain were identified as the prevailing etiological agents of an outbreak of acute respiratory infection that occurred among young children in Lisbon in 2004 and that resulted in two deaths. Our data provide new evidence that recombination drives the generation

of highly virulent human adenovirus strains with epidemic and lethal potential.

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