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J Clin Microbiol. 2005 Dec;43(12):6176-82.

MOLECULAR IDENTIFICATION OF ADENOVIRUSES IN CLINICAL SAMPLES BY ANALYZING A PARTIAL HEXON GENOMIC REGION

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Running title: ADENOVIRUS TYPING BY PARTIAL HEXON SEQUENCING

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Key words: adenovirus typing, molecular characterization, clinical microbiology.

ABSTRACT

Here we present a system for adenovirus detection and genotyping based on PCR amplification and phylogenetic analysis of a conserved hexon gene fragment. The system was validated using 157 sequences (86 previously typed and 71 clinical samples) and correctly identified species and serotype in 100% and 84% of sequences respectively. Clinical correlation yielded known and novel associations between specific serotypes and clinical presentation.

Human adenoviruses (HAdVs) cause a wide range of clinical syndromes and are being increasingly recognized in cases of severe or fatal pneumonia, hemorrhagic cystitis, hepatitis, or disseminated disease in pediatric bone marrow transplant recipients (15). HAdVs are classified into six species, A to F, comprising 51 serotypes (7). Serotype identification is critical for epidemiological surveillance, detection of new strains, assessment of treatment efficacy and understanding HAdV pathogenesis (19).

Molecular typing methods have been established to circumvent practical problems associated with traditional serum neutralization studies (1-4, 12, 13, 16, 17, 25, 26). Molecular methods also have disadvantages; restriction fragment length polymorphism analysis of adenoviral DNA may fail if mutations are present within the restriction site and multiplex PCR assays are currently not able to discriminate between serotypes (21, 30). PCR amplification of the hypervariable portion of the hexon gene followed by DNA sequencing has recently been proposed as a typing method; however, this method was unable to discriminate between species B and E and was validated with only 10 clinical samples (25).

We have previously detected HAdV infection in clinical samples using generic HAdV primers in singleplex (6) and multiplex assays; these assays have been extensively validated and used routinely for clinical diagnosis (8, 10). Here we report that DNA sequencing and phylogenetic analysis of this moderately conserved region (aa 540 to 662) of the hexon gene (11) is sufficient to allow HAdV speciation and, in most cases, serotype identification. We have confirmed and also noted new associations between specific serotypes and clinical presentations.

HAdV infection was detected by generic PCR in 46 clinical specimens and 25 HAdV culture isolates sent for diagnostic evaluation at Centro Nacional de Microbiologia, ISCII, Spain; clinical materials, patient characteristics and alternate methods to detect HAdV infection, such as cell culture, direct immunofluoresence assay and latex agglutination, are listed in Table 1.

For comparison, 47 prototype HAdV strains, each representing a distinct serotype, were obtained from the American Type Culture Collection (Manhassas, VA) or from an existing collection in our institute (Table S1).

Nucleic acids were extracted (9) and the singleplex PCR assay performed as previously described (6). Briefly, 5 μl of the nucleic acid extraction were added to 45 μl of reaction mixture containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 500μM each dNTP, 4 mM $MgCl_2$, 2.5 units of Taq polymerase (Amplitaq; Perkin-Elmer Cetus, Norwalk) and 20 pmol of degenerate primers ADHEX1F (5'CAACACCTAYGASTACATGAA3') and ADHEX1R (5'KATGGGGTARAGCATGTT3'). Temperature and time profiles were: 94ºC for 1 min, 50ºC for 1 min and 68ºC for 1 min for 30 cycles, and a final incubation at 68ºC for 5 min. Amplification products (475 bp) from this PCR reaction were visualized by agarose gel electrophoresis and sequenced directly. For clinical samples where direct sequencing was not possible due to low DNA yield, two independent nested reactions were performed

with 20 pmol of degenerate primer ADHEX2F (5'CCCITTYAACCACCACCG3') and ADHEX1R or 20 pmol of ADHEX1F and ADHEX2R (5'ACATCCTTBCKGAAGTTCCA3'). Amplified products were purified and sequenced in both directions using an automated ABI PRIMS 377 model sequencer. The sequences were deposited in the GenBank sequence database under accession numbers AY819809 to AY819926.

The consensus sequence was compared and aligned against other sequences from samples or the DNA database using the program CLUSTAL X (version 1.83). The relationships between individual viruses were established using Neighbour-joining, UPGMA, and nucleotide substitution methods (Tamura-Nei, Kimura-2p, Jukes-Cantor). Phylogenetic trees were reconstructed through the Neighbour-joining method (MEGA package, version 3) by 1000 times bootstrap re-sampling. Pairwise comparisons were also made by global alignment using the Needleman Wunsch algorithm (20), implemented by a program from EMBOSS (22).

The phylogenetic tree showed 6 different clusters representing species A to F at the nucleotide (Figure 1) and amino acid levels with bootstrap values ranging from 59 to 99. Results obtained when the 46 clinical samples and the 25 HAdV isolates were compared with sequence of the reference strains are presented in Table 2. All clinical samples were speciated, and 42 (91%) of 46 were serotyped. All 25 isolates were speciated, and 22 (88%) of 25 were serotyped. Phylogenetic analysis revealed subclusterings, except in species E, D and B2; serotype HAdV-7 could be separated in to two lineages (17). Serotypes of species D were not clearly discriminated because of high homology. Serotypes 11, 34 and 35 of subgroup B2 were indistinguishable. HAdV-4 is the only member of species E. The Needleman Wunsch pairwise algorithm produced identical results to those obtained via phylogenetic analyses (Table 2 and S2). Although both analyses permitted accurate HAdV classification, pairwise similarity analysis has the advantage of speed and simplicity.

Using our method, we found new associations between specific clinical syndromes and HAdV serotypes (Table 3). Typically, respiratory tract diseases are associated with species B1, C and E, gastrointestinal diseases with species A and F, eye diseases with species D and E, and kidney and urinary tract diseases with species B2.

Acute respiratory diseases due to HAdV are attributed primarily to serotypes 3, 4, 7, 14 and 21 (species B and E) (17, 40 36, 37). We observed serotypes 1, 2, 5, and 6 (species C) and species D. Pneumonia in children has been associated with serotypes 1-3 and 7, whereas pneumonia in adults is predominantly associated with serotypes 4 and 7. We also found serotypes 5 and 6 in young children, and subgroup B2 in immunosuppressed patients.

Gastrointestinal manifestations of HAdV infection include diarrhea and hepatitis. In addition to serotypes 40 and 41 (4, 23, 28), we identified cases of diarrhea associated with serotypes 6, 12, 16, 31, and a member of species D. The significance of these findings is not clear as members of species C may

be excreted in faeces during subclinical infection. HAdV hepatitis has been reported in children recipients of liver transplants associated with serotypes 1, 2 and 5 (species C); in this study we detected the remaining member of species C, serotype 6, in a case of fatal hepatitis.

Epidemic keratoconjunctivitis has been associated with serotypes 8, 19 and 37 (species D) and serotype 11 (species B2). Our sequences revealed not only serotype 8 (species D), but also serotypes 4 and 7 (species E and B1, respectively). Adenovirus 4 (species E) can cause either respiratory or mild ocular infections (29) and nosocomial epidemic conjunctivitis in Japan (5, 27).

Cases of acute hemorrhagic cystitis in young children have been associated with species B2 serotypes 11 and 21 and fatal infections due to species B1, serotypes 3 and 7, have been reported (14, 18, 23, 31). In our study, this disease is associated with B1 serotypes 16 and 14 (species B1 and B2), and an indistinguishable member of the cluster 11, 34, 35 (species B2). The fact that subgroups B1 and B2 use different cellular receptors for viral entry underscores the importance of typing HAdV for epidemiology and pathobiology (24).

Finally, we found serotypes 4 and 5 (species E and C) in throat swabs of 4 patients with fever, morbilliform rash, Koplik's spots, and cough who had a history of MMR vaccination and were negative for measles and rubella. To our knowledge this is the first report of HAdV presenting as a syndrome compatible with measles infection.

Using the database and classification system from this study, we have deployed a website (www.greeneidlab.columbia.edu) wherein clinical laboratories can submit hexon sequences to generate an automatic report detailing the serotype, date and location of the most similar sequence isolate in the database. This system will allow new genotypes to be readily identified because the classification scheme will fail to relate them to any described serotype. Epidemiological surveillance of HAdV serotypes will improve our understanding of the global burden of HAdV infection. High-throughput systems described here will facilitate HAdV surveillance and enhance understanding of HAdV pathogenesis.

ACKNOWLEGMENTS

The authors acknowledge technical assistance from Manuela López-Valero in the Respiratory Viruses Laboratory, CNM, ISCIII, Majadahonda, Madrid, Spain. We thank Neil Renwick for his critical reviewing of the manuscript and helpful comments. This work was supported by grants from the National Institutes of Health (AI51292, AI55466, AI056118, U54AI057158), the Ellison Medical Foundation and ISCIII (MPY 1251/00 and MPY 1018/04).

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FIGURE LEGENDS

Figure 1. Nucleotide sequences were aligned with Clustal W. Phylogenetic analyses were performed using the Kimura two-parameter model as a model of nucleotide substitution and using the neighbour joining method to reconstruct phylogenetic trees (MEGA version 2.1 software package). The statistical significance of the phylogenies constructed was estimated by bootstrap analysis with 1,000 pseudoreplicate data sets.

Table 1. Summary of patient data including clinical syndrome, sample type, subject age, epidemiology, cell cultures and direct antigen detection results.

Table 2. Typing of HAdV: comparison of phylogenetic and pairwise alignment methods.

Table 3. Summary of the HAdV serotype infections associated with disease or persistence.

PROPOSED SUPPLEMENTARY MATERIAL

Table S1. Adenovirus nucleotide sequences of reference or wild type strains from GenBank database.

Table S2. Exhaustive results showing the score values obtained typing HAdV sequences collected from the GenBank.

TABLE 1.

Sample[a]	Syndrome	Patient sample	Patient age	Epidemiology	Cell Culture	Antigen detection[b]	
						IFA	LA
SO3360_01	Bronchiolitis	NPA	1,6 yr	sporadic	cont	-	ND
SO3790_03	Bronchiolitis	NPA	<5yr	sporadic	+	+	ND
SO4010_04	Bronchiolitis	NPA	1,1 yr	sporadic	-	-	ND
SO4363_04	Bronchiolitis	NPA	10 mo	sporadic	ND	-	ND
SO4366_04	Bronchiolitis	NPA	11 mo	sporadic	ND	-	ND
SO4390_04	Bronchiolitis	NPA	<5yr	sporadic	cont	+	ND
SO4405_04	Bronchiolitis	NPA	<5yr	sporadic	+	+	ND
SO4408_04	Bronchiolitis	NPA	<5yr	sporadic	ND	-	ND
SO4425_04	Bronchiolitis	NPA	1,5 yr	sporadic	+	+	ND
SO4427_04	Bronchiolitis	NPA	<5yr	sporadic	+	+	ND
SO4429_04	Bronchiolitis	NPA	<5yr	sporadic	-	-	ND
G1066_01	Influenza-like	TS	33 yr	sporadic	-	-	ND
G1108_01	Influenza-like	TS	26 yr	sporadic	-	-	ND
G1253_02	Influenza-like	TS	2 yr	sporadic	-	-	ND
G1507_04	Influenza-like	TS	33 yr	sporadic	-	ND	ND
G1508_04	Influenza-like	TS	25 yr	sporadic	-	-	ND
G1634_04	Influenza-like	TS	29 yr	sporadic	-	-	ND
R1625_04	Pneumonia	NW	20 d	sporadic	ND	ND	ND
1184X_93	Pneumonia	BAL	<5yr	sporadic	+	ND	ND
R1629_04	HIV+, Pneumonia	BAL	30 yr	sporadic	-	ND	ND
591Fi_01	LRI	BAL		sporadic	-	ND	ND
992Fi_01	LRI	BAL		sporadic	-	ND	ND
593Fi_01	LRI	BAL		sporadic	-	ND	ND
130C_99	LRI	NW	19 mo	sporadic	ND	ND	ND
R1612_04	BMTx, Hemorrhagic cystitis	NW	14 yr	sporadic	-	ND	ND
226I_03	BMTx, Hemorrhagic cystitis	Urine	40 yr	sporadic	ND	ND	ND
1938I_89	BMTx, Hemorrhagic cystitis	Urine	30 yr	sporadic	+	ND	ND
1370I_03	BMTx, Hemorrhagic cystitis	Urine	15 yr	sporadic	ND	ND	ND
411I_04	LITx, Fatal Hepatitis	Liver N	5 yr	sporadic	ND	ND	ND
1803F_03	Exanthema, fever	TS	3 yr	sporadic	ND	ND	ND
2549F_03	Exanthema, fever	TS	4 yr	sporadic	ND	ND	ND
1804F_03	Exanthema, fever	TS	6 yr	sporadic	ND	ND	ND
1292F_01	Exanthema not fever	TS	2 yr	sporadic	+	ND	ND
824O_02	Keratoconjunctivitis	Eye swab	56 yr	sporadic	ND	ND	ND
928O_04	Keratoconjunctivitis	Eye swab	30 yr	Out-Jaén	ND	ND	ND
926O_04	Keratoconjunctivitis	Eye swab	55 yr	Out-Jaén	ND	ND	ND
925O_04	Keratoconjunctivitis	Eye swab	61 yr	Out-Jaén	ND	ND	ND
D0005_01	Diarrhea	Feces	<14 yr	sporadic	-	ND	+
D0007_01	Diarrhea	Feces	<14 yr	sporadic	-	ND	+
D0001_01	Diarrhea	Feces	<14 yr	sporadic	-	ND	+
D0011_01	Diarrhea	Feces	<14 yr	sporadic	-	ND	+
D0002_01	Diarrhea	Feces	<14 yr	sporadic	-	ND	+
D0004_01	Diarrhea	Feces	<14 yr	sporadic	-	ND	+
D0015_01	Diarrhea	Feces	<14 yr	sporadic	-	ND	+
D0016_01	Diarrhea	Feces	<14 yr	sporadic	-	ND	+
D0017_01	Diarrhea	Feces	<14 yr	sporadic	-	ND	+
R1650_04	BMTx, Diarrhea	Feces	<5yr	sporadic	Hep-2	+	ND
R1641_04	BMTx, Diarrhea	Feces	<5yr	sporadic	Hep-2	+	ND
C1640_03	BMTx, Diarrhea	Feces	6yr	sporadic	HEF	+	ND
R1647_04	BMTx, Diarrhea	Feces	<5yr	sporadic	Hep-2	+	ND
C5335_01	BMTx, pneumonia	BAL+Biopsy	49yr	sporadic	HEF	+	ND
594_89	HIV+, Diarrhea	Feces	>40yr	sporadic	Hep-2	+	ND
4030_96	Influenza-like	Feces	3yr	sporadic	Hep-2	+	ND
C1662_03	Bronchiolitis	NPA	5mo	sporadic	HEF	+	ND
C1491_02	Bronchiolitis	NPA	1mo	sporadic	HEF	+	ND
C1519_02	Bronchiolitis	NPA	15mo	sporadic	HEF	+	ND
C1201_00	Bronchiolitis	NPA	8mo	sporadic	HEF	+	ND
G3093_03	LRI	Feces	30 yr	sporadic	Hep-2	+	ND
C1167_00	LRI	NPA	3mo	sporadic	HEF	+	ND
859_96	Keratoconjunctivitis	Eye swab	>40yr	out-Tenerife	Hep-2	+	ND
860_96	Keratoconjunctivitis	Eye swab	>40yr	out-Tenerife	Hep-2	+	ND
519_93	Keratoconjunctivitis	Eye swab	>40yr	out-Toledo	Hep-2	+	ND
856_96	Keratoconjunctivitis	Eye swab	>40yr	out-Tenerife	Hep-2	+	ND
615_96	Keratoconjunctivitis	Eye swab	>40yr	out-Pamplona	Hep-2	+	ND
636_96	Keratoconjunctivitis	Eye swab	>40yr	out-Pamplona	Hep-2	+	ND
647_96	Keratoconjunctivitis	Eye swab	>40yr	out-Pamplona	Hep-2	+	ND
841_94	Keratoconjunctivitis	Eye swab	>40yr	sporadic	Hep-2	+	ND
43024_02	Keratoconjunctivitis	TS	>40yr	out-Madrid	HEF	+	ND
C4292_02	Keratoconjunctivitis	Eye swab	>40yr	out-Madrid	HEF	+	ND
C1629_02	newborn control	NPA	0d	sporadic	HEF	+	ND
G1T4_03	environmental water	---	---	----	A549	+	ND

[a] Clinical sample ID_year and Isolate_year (boldface).

[b]Cells were collected and stained by standard methods. In respiratory samples HAdV infection was detected by direct IFA with monoclonal antibodies (Chemicon, Temecula, CA). The IFA was carried out with fluorescein isothiocyanate FITC)-conjugate goat anti-mouse IgG (Sigma). Monoclonal antibodies for detection of
fusion protein (F0/F1) of all strains of parainfluenza virus type 1, and haemagglutinin of all strains of parainfluenza viruses types 2 and 3, for enteroviruses were also obtained from Chemicon.. All specimens were collected in 3 ml of virus transport medium (MEM, Gibco-BRL, Life Technologies, Paisley, Scotland; penicillin 200U/ml, and streptomycin 200 mg/ml, BioWhittaker, MA; mycostatin 200U/ml, Sigma; bovine serum albumin 0.25%, Merck, Darmstadt, Germany). Stool samples were tested for HAdV (HAdV-40 and -41) by latex agglutination (Adenolex, Orion, Helsinki, Finland)

Abbreviations: BMT, bone marrow transplant; LITx, liver and intestinal transplant; NPA, nasopharyngeal aspirates; TS, throat swab; BAL, bronchoalveolar lavage; NW, nasal wash; LA, latex agglutination; cont, contamination of cell cultures; ND, not done; Out-, outbreak; HEF: human embryonic fibroblast cell lines; BMTx, bone marrow transplant.

TABLE 2.

Name	Typing by phylogeny			Typing by pairwise alignment	
	Species[a]	Bootstrap value	Serotype[b]	NW Homology Score (%)[b]	Serotype
Clinical Samples					
G1066_01	C	79	HAdV-2	98,5	HAdV-2
SO4010_04	C	79	HAdV-2	100,0	HAdV-2
G1634_04	B	74	HAdV-3	99,0	HAdV-3
R1625_04	B	74	HAdV-3	99,0	HAdV-3
SO4425_04	B	74	HAdV-3	99,0	HAdV-3
SO4429_04	B	74	HAdV-3	99,0	HAdV-3
G1108_01	E	99	HAdV-4	93,1	HAdV-4
1292F_01	E	99	HAdV-4	95,6	HAdV-4
1803F_03	E	99	HAdV-4	93,1	HAdV-4
SO3360_01	C	99	HAdV-5	96,1	HAdV-5
SO3790_03	C	99	HAdV-5	100,0	HAdV-5
SO4366_04	C	99	HAdV-5	96,1	HAdV-5
591Fi_01	C	99	HAdV-5	99,5	HAdV-5
992Fi_01	C	99	HAdV-5	99,5	HAdV-5
130C_99	C	99	HAdV-5	99,5	HAdV-5
2549F_03	C	99	HAdV-5	99,0	HAdV-5
1804F_03	C	99	HAdV-5	98,5	HAdV-5
G1507_04	C	59	HAdV-6	99,0	HAdV-6
SO4408_04	C	59	HAdV-6	99,5	HAdV-6
SO4427_04	C	59	HAdV-6	99,5	HAdV-6
G1508_04	C	59	HAdV-6	99,0	HAdV-6
593Fi_01	C	59	HAdV-6	98,5	HAdV-6
1370I_03	C	59	HAdV-6	99,5	HAdV-6
411I_04	C	59	HAdV-6	99,5	HAdV-6
D0016_01	C	59	HAdV-6	99,5	HAdV-6
SO4363_04	C	59	HAdV-6	99,1	HAdV-6
SO4405_04	B	98	HAdV-7 genotype 2	99,5	HAdV-7 genotype 2
G1253_02	B	98	HAdV-7 genotype 2	99,5	HAdV-7 genotype 2
1184X_93	B	98	HAdV-7 genotype 2	99,5	HAdV-7 genotype 2
824O_02	B	98	HAdV-7 genotype 2	99,0	HAdV-7 genotype 2
R1629_04	B	61	HAdV-11, 34 or 35	98,2*	HADV-11, 34 or 35
R1612_04	B	61	HAdV-11, 34 or 35	97,8*	HADV-11, 34 or 35
1938I_89	B	61	HAdV-11, 34 or 35	99,2*	HADV-11, 34 or 35
226I_03	B	71	HAdV-14	96,6	HAdV-14
D0002_01	F	99	HAdV-40	100,0	HAdV-40
D0004_01	F	99	HAdV-40	98,5	HAdV-40
D0017_01	F	99	HAdV-40	100,0	HAdV-40
D0015_01	F	99	HAdV-40	100,0	HAdV-40
D0005_01	F	99	HAdV-41	97,0	HAdV-41
D0007_01	F	99	HAdV-41	98,0	HAdV-41
D0001_01	F	99	HAdV-41	97,0	HAdV-41
D0011_01	F	99	HAdV-41	98,0	HAdV-41
928O_04	D	94	NIB; Species D (not HAdV-8)	95,5*	Species D (not HAdV-8)
926O_04	D	94	NIB; Species D (not HAdV-8)	95,6*	Species D (not HAdV-8)
925O_04	D	94	NIB; Species D (not HAdV-8)	95,6*	Species D (not HAdV-8)
SO4390_04	D	94	NIB; Species D (not HAdV-8)	95,0*	Species D (not HAdV-8)
HAdV isolates					
C1519_02	C	95	HAdV-1	99,0	HAdV-1
C1662_03	C	95	HAdV-1	99,0	HAdV-1
C1491_02	C	95	HAdV-1	99,5	HAdV-1
C1629_02	B	74	HAdV-3	99,0	HAdV-3
4030_96	B	74	HAdV-3	99,0	HAdV-3
859_96	E	99	HAdV-4	92,2	HAdV-4
860_96	E	99	HAdV-4	89,4	HAdV-4
519_93	E	99	HAdV-4	90,7	HAdV-4
856_96	E	99	HAdV-4	92,7	HAdV-4
C1201_00	C	59	HAdV-6	100,0	HAdV-6
G3093_03	C	59	HAdV-6	99,0	HAdV-6
615_96	D	99	HAdV-8	100,0	HAdV-8
636_96	D	99	HAdV-8	100,0	HAdV-8
647_96	D	99	HAdV-8	100,0	HAdV-8
841_94	D	99	HAdV-8	100,0	HAdV-8
43024_02	D	99	HAdV-8	98,0	HAdV-8
C4292_02	D	99	HAdV-8	98,5	HAdV-8
R1650_04	A	99	HAdV-12	97,5	HAdV-12
R1641_04	A	99	HAdV-12	97,5	HAdV-12
C5335_01	B	99	HAdV-21	96,5	HAdV-21
R1647_04	B	71	HAdV-16	99,5	HAdV-16
C1640_03	A	99	HAdV-31	98,5	HAdV-31
G1T4_03	D	94	NIB; Species D (not HAdV-8)	97,0*	Species D (not HAdV-8)
594_89	D	94	NIB; Species D (not HAdV-8)	96,1*	Species D (not HAdV-8)
C1167_00	D	94	NIB; Species D (not HAdV-8)	96,6*	Species D (not HAdV-8)

[a] species identified by bootstrap;
[b] NIB: serotype not identified by bootstrap;
[c] *Average score from all members of the group. Gray shading, when the NW score for the second highest serotype was higher than 93 %. Due to their close relationship, all cases of HAdV-2 and -6 present this situation. In any way, in all cases the highest score pointed to the correct serotype.

Table 3. Summary of the HAdV serotype infections associated with disease or persistence

Disease		Individuals most at risk	Principal serotypes reported (16)	Found in this work
Acute febrile pharyngitis		Infants, young children	1-3, 5-7	
Acute respiratory disease		military recruits	3, 4, 7, 14, 21	1, 2, 3, 4, 5, 6, 7, species D
Pneumonia		Infants, young children	1-3, 7	3, 5, 6, 7, (11, 34, 35)[a], 21
		military recruits	4, 7	
Pharyngoconjunctival fever		school-aged children	3, 7, 14	
Epidemic keratoconjunctivitis		any age group	8, 11, 19, 37	4, 8, 7, species D
Acute hemorrhagic cystitis		young children	11, 21	(11,34,35)[b], 6[b], 14[b]
Gastroenteritis		Infants, young children	40, 41	6, 12[b], 16[b], 31[b], 40, 41, species D[a]
Hepatitis		infants and children with liver transplants	1, 2, 5	6[d]
Exanthema			Not reported previously	4,5
Persistence:	in urinary tract	immunosuppressed patients	34, 35	
	in colon		42-49	

[a] HIV positive
[b] Bone marrow transplant.
[c] Liver and intestinal transplant, fatal case.