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Originally published as:

**Chmielewicz, B., Benzler, J., Pauli, G., Krause, G., Bergmann, F., Schweiger, B.
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(2005) Journal of Medical Virology, 77 (2), pp. 232-237.**

Respiratory disease caused by a species B2 *Adenovirus* in a military camp in Turkey

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Running Title: Species B2 *Adenovirus* ARD in Turkey

<u>Abstract</u>

In April 2004, two patients were admitted to hospital in Berlin, Germany, with clinical signs of acute respiratory infection after having returned from a military exercise in their home country of Turkey. They were admitted to a high security infectious disease unit as epidemiological data pointed to an outbreak of unknown etiology. Samples taken at the time of admission proved to be strongly positive for *Adenovirus* by PCR, but negative for *Influenza A/H1N1 virus*, *Influenza A/H3N2 virus, Influenza B virus*, *Respiratory syncytial virus*, and *SARS coronavirus*. No evidence for a bacterial infection was obtained by serological tests and blood cultures. The adenovirus detected was characterized further by genotyping and was identified as a species B2 virus with the highest similarity to adenovirus type 11a.

Introduction

The *Adenovirus* family comprises double-stranded DNA viruses that have a common nonenveloped icosahedral capsid morphology and a similar genome structure. The 51 serotypes infecting humans are grouped into six species (A-F) within the genus *Mastadenovirus*, based on biological characteristics and DNA-sequence properties [Fields et al., 1996; Walls et al., 2003]. The species B viruses are divided further into two subspecies, named B1 and B2. Adenoviruses are a common cause of infections of the gastrointestinal, urinary or respiratory tract, and are isolated most frequently from young children [Gu et al., 2003; Schmitz et al., 1983; Walls et al., 2003].

Since the serotypes that are grouped within one species are highly homologous in their DNA sequence, they are considered to share similar tropism, pathogenic potential and epidemiological features [Allard et al., 2001; Kidd et al., 1996; Schmitz et al., 1983; Xu et al., 2000]. Species A and F viruses are isolated regularly from children with gastrointestinal disease [Heim et al., 2003; McIver et al., 2001; Schmitz et al., 1983; Uhnoo et al., 1990; Wilhelmi et al., 2003], while respiratory disease is usually caused by viruses belonging to species B1, C or E [Heim et al., 2003; Schmitz et al., 1983]. In contrast, species B2 viruses have been isolated most commonly from the urinary tract, especially from patients with an impaired immune function [Basler and Horwitz, 1996; Heim et al., 2003; Mei et al., 2003; Segerman et al., 2003], and are reported only rarely in the context of other clinical symptoms such as respiratory disease or epidemic conjunctivitis in immunocompetent patients [Hierholzer et al., 1974; Klinger et al., 1998].

In adults, epidemics of adenovirus-associated disease have been described repeatedly in military recruits. These patients suffer from acute respiratory disease,

which is usually caused by the adenovirus serotypes 4 or 7 (species E and B1) [Blasiole et al., 2004; Faix et al., 2004; Heim et al., 2003; Kolavic-Gray et al., 2002; Lauer et al., 2004]. A presumed outbreak of respiratory disease is described in a military camp in Turkey that was probably caused by an adenovirus belonging to species B2.

Materials and Methods

Epidemiological investigation

The description of the outbreak is based largely on concordant statements of the two patients and an independent interview with a third participant in the military training, who had not been admitted to hospital. The Turkish Embassy in Germany and the German Foreign Service (Auswärtiger Dienst) in Turkey were contacted for further information and internationally accessible publications in the Turkish press were screened.

Clinical samples

The clinical samples were collected from the two patients at the time of admission to hospital in April 2004. From each patient, a sputum sample was taken; additionally, a urine sample was obtained from one of the two patients. The sputum was filled up to 5 ml with cell culture medium before processing further; the urine was used without any dilution.

Cell Culture

Monolayer cultures of A549, HeLa, HEp2, CaCo2 and MDCK cells were infected with 200µl of the diluted and sterile filtered sputum samples and maintained in MEM/HEPES (containing 2% FKS, 100 U/ml Penicillin G and 100 µg/ml streptomycin sulfate) at 37°C in a closed system without CO_2-incubation. For up to six weeks, cells were examined daily with regard to a cpe (cytopathogenic effect), and passaged if necessary. The supernatant of the cultures was incubated on freshly seeded cells.

Preparation of nucleic acids and cDNA synthesis

DNA and RNA were extracted simultaneously from 200 µl of the clinical samples using the RTP DNA/RNA Virus Mini Kit (Invitek). The nucleic acids were eluted in 60 µl of elution buffer.

cDNA was prepared with random hexamer primers according to standard procedures using the Superscript RT enzyme (Invitrogen) and RNasin (Promega).

Conventional PCR

PCR reactions were set up in a total volume of 25 µl containing 1x PCR buffer, 2 mM $MgCl_2$, 1 mM dNTPs with dUTP, 500 nM of each primer (listed in Table 1), 0.5 U Platinum Taq Polymerase (Invitrogen) and 1-10 µl template DNA. Amplification was carried out in a GeneAmp 2400 or 9600 Instrument (Applied Biosystems) for a total of 40 cycles. After an initial denaturation step at 95°C for 5 minutes, each cycle consisted of denaturation at 95°C for 30 seconds, followed by annealing at a primer-specific temperature for 30 seconds and primer extension at 72°C for a time span depending on the amplicon length (1 min/1.000 bp). The PCR product was analyzed on a 1-2% agarose gel and visualized with ethidium bromide under ultraviolet light.

The Combizyme DNA Polymerase Mix (Invitek) was used for the amplification of long PCR products (P-060, P-061) as suggested by the manufacturer.

Nested PCR

For nested amplification, reaction conditions were as described for conventional PCR. 2 µl of the first round product served as a template for the second round PCR. In both rounds, 40 cycles were carried out.

Quantitative PCR

Quantitative real-time PCR was carried out using an ABI Prism 7700 or 7900 Sequence Detection System (Applied Biosystems) in a total reaction volume of 25 µl. For the detection of *Influenza A/H1N1 virus*, *Influenza A/H3N2 virus, Influenza B virus*, *Respiratory Syncytial virus*, *SARS Coronavirus* and adenovirus hexon the reaction conditions were as described elsewhere [Schweiger et al., 2000]. For adenovirus Dpol amplification and subsequent genotyping, the reaction conditions were as described in [Chmielewicz et al., 2005].

Sequence Determination

Amplicons were sequenced directly after purification (PCR Purification Kit, QIAGEN) using the dye terminator chemistry (ABI-Prism Big Dye Terminators v3.1 Cycle Sequencing Kit, Applied Biosystems) in an ABI-Prism 3100 Genetic Analyzer (Applied Biosystems). The sequences obtained were analyzed, assembled and aligned using the DNA STAR Software Package. All new sequences described in this study were determined by sequencing at least two independent PCR products in both directions, i.e. each nucleotide has been determined at least four times.

The neighbor-joining method of the PHYLIP program package [Felsenstein, 1989] was used for phylogenetic analysis.

Accession Numbers

All sequences were deposited in the GenBank Databases of the National Center of Biotechnology Information under the following Accession Numbers: hexon gene (AY972815), fiber gene (AY972816). The amino acid sequences of these residues were deduced.

Results

Clinical Description

In April 2004, two 37- and 39-year-old men returned to Berlin, Germany, from a four week military training in Burdur (approximately 120 km north of Antalya, Turkey) with severe respiratory symptoms. They were admitted to hospital immediately to a high security infectious disease unit with negative air pressure rooms. Strict barrier nursing was implemented as an outbreak of unknown origin or a bioterrorism attack could not be ruled out at presentation.

Both patients presented with an unproductive cough, constipation and temperature around 38°C, but were otherwise in fairly good general condition. Physical examination was unremarkable. Thorax X-ray and oxygen levels were normal. Laboratory testing revealed increased levels of CK, transaminases and CRP (16 to 20 x ULN), but no leucocytosis. Elevated CK levels (808 U/l and 444 U/l) could not be explained by excessive physical training in the last two weeks, and returned to normal at discharge. In one patient CKmB was elevated (25 U/l), but no abnormalities were seen in ECGs.

A mild form of atypical pneumonia was suspected and serological testing was done for *Coxiella*, *Rickettsia*, *Mycoplasma*, *Chlamydia*, *Legionella*, *Brucella* and typhoid fever. Routine blood cultures as well as testing for viral pathogens in sputum and urine samples were undertaken. Antibiotic therapy with chinolones was started.

After virological tests led to a definitive diagnosis, measures were reduced to standard isolation. Both patients recovered quickly under symptomatic treatment and were released home after some days of observation in a general ward.

Epidemiological data

According to the patients, about 2250 men aged 18 to 40 years living in more than 50 countries participated in the training. Of these, hundreds fell ill with similar symptoms (with varying degrees of severity) and many were treated at a local hospital. A 26-year-old man, said to be from Canada, died on April 25th 2004, but it remained unclear in which way his death was linked to the outbreak.

Approximately half the participants came from Germany. Others were from Switzerland, Portugal, Belgium, the Netherlands, USA and several Asian and Arab countries. The participants were accommodated in groups of 40, while lavatory facilities and a canteen were shared by about 400 people. Instruction meetings were attended by up to 1000 people.

The outbreak of respiratory illness started toward the end of their first week of stay and spread continuously over the following two weeks with more than hundred recruits falling ill daily at the height of the outbreak. Most participants continued attendance at meetings and exercises despite clinical signs of illness. About 2000 teachers completing their basic military training as well as 1000 supervisors were also present on the same campus, but seemingly they were not affected by the outbreak in a similar way as the expatriate participants.

Before his departure to Germany, one of the patients was advised to carry a mask during air travel and contact a doctor after arrival in Berlin. Both patients had only few contacts with other people before being hospitalized.

The surveillance unit of the Robert Koch Institute (RKI), the central federal institution responsible for disease control and prevention in Germany, contacted the Turkish

authorities, who confirmed the death case, but could not provide details regarding its cause. The outbreak has neither been confirmed nor denied officially; thus, the description is based solely on concordant statements of the two patients and a third returning participant.

The surveillance unit of the Robert Koch-Institut informed German and international health authorities and the WHO about this outbreak, because the cause had not yet been established at that time and numerous countries may have been involved. However, no confirmation of further cases with similar clinical picture and epidemiological link to the outbreak was received.

Results of bacteriological and virological tests

Serologically, no infection with a bacterial pathogen causing pneumonia (*Coxiella*, *Rickettsia*, *Mycoplasma*, *Chlamydia*, *Legionella*, *Brucella* and typhoid fever) became evident. Also, routine blood cultures were negative.

Three samples were taken for virological examination (sputum of patient 1, sputum and urine of patient 2). Both specimens were tested by PCR for several viral pathogens causing severe respiratory disease. No amplification was observed for *Influenza A/H1N1 virus*, *Influenza A/H3N2 virus, Influenza B virus*, *Respiratory syncytial virus*, and *SARS coronavirus* in either sample, while both sputa proved to be strongly positive for adenovirus using an unpublished real-time PCR (adenovirus hexon). To confirm this result, a PCR-based assay for generic adenovirus detection and genotyping (Adenovirus DPol PCR) was applied to the samples [Chmielewicz et al., 2005]. Again, the two sputum samples were strongly positive. Adenovirus DNA was not detected in the urine by either PCR assay.

Molecular analyses of the adenoviral strain

First, the adenovirus DPol PCR amplicons which were obtained were genotyped by melting analysis [Chmielewicz et al., 2005]. Both sputa contained viruses belonging to adenovirus species B2. This result was confirmed by sequencing of several regions of the viral genome after amplification from the samples. First, a set of nested primers was used to amplify the complete hexon gene (P-060, P-061). The resulting amplicons were sequenced in a 1435 bp stretch comprising the seven hypervariable regions (HVR) of this gene [nt 18361 − 19801 of adenovirus 11p (AF532578), all primers listed in Table 1]. Both sequences were completely identical to each other and most highly homologous to a partial hexon sequence of a clinical adenovirus 11 isolate [strain 91-038T, accession number AB162772, Identities = 1195/1196 (99%)], but showed a lower grade of homology to the adenovirus 11 prototype strain [Slobitski strain (adenovirus 11p), accession number AF532578, Identities 1403/1441 (97%), Gaps 6/1441 (0%)]. The same was observed for the protein sequence deduced (Table 2 and Fig. 1).

Furthermore, a 581 bp fragment of the fiber gene [nt 30906 − 31486 of adenovirus 11p (AF532578)] spanning the 3'- part of the tail to the 5'-part of the knob region was amplified and sequenced using primers P-016 [Xu et al., 2000]. Again, both sequences were completely identical. They showed the highest homology to an adenovirus 11a fiber sequence (accession number L08232) with only one nucleotide exchange, but a lower homology to the Slobitski strain (Table 2 + Fig. 2).

Discussion

Although adenoviruses primarily cause disease in young children [Schmitz et al., 1983], it has been reported repeatedly that these viruses also cause acute respiratory disease in adults [Hierholzer et al., 1974; Schmitz et al., 1983]. These outbreaks usually occur in military training facilities and are characterized by respiratory tract disease with extensive morbidity (up to 80%) and occasional mortality [Faix et al., 2004; Gray et al., 2000; Kolavic-Gray et al., 2002; Lauer et al., 2004]. The serotypes causing the vast majority of outbreaks were adenovirus types 4 and 7, followed by types 3 and 21 [Gray et al., 2000; Kolavic-Gray et al., 2002].

The acute respiratory disease that was observed among attendees of a military training camp in Burdur, Turkey, was probably also caused by an adenovirus. The presence of large amounts of adenovirus DNA in the sputum samples of both patients and absence of any other relevant pathogen suggests this virus to be responsible for the presumed outbreak. This assumption is reinforced by the patients' clinical course and the accessible epidemiological data which aligns well with previous reports on adenovirus-associated acute respiratory disease in military camps [Barraza et al., 1999; Gray et al., 1999; Hendrix et al., 1999; Hierholzer et al., 1974; Kolavic-Gray et al., 2002]. The spread of the virus was favoured by the fact that participants continued to attend training in spite of being sick, presumably because sick-leave might have resulted in the training being prolonged or repeated, since this type of short course military training is mandatory for male expatriate Turkish citizens who want to acquire a different nationality.

Although the fresh sputum samples from both patients were incubated with several cell lines that are permissive for adenovirus infection, we unfortunately did not

succeed in cultivating the virus. Therefore, a further characterization of the virus could only be carried out by molecular analyses.

For this purpose, several parts of the viral genome were sequenced. All sequences obtained from both sputum samples proved to be completely identical to each other. Therefore, both patients were infected with the same virus. To allow for the determination of the viral serotype, a 1435 bp fragment of the hexon gene was sequenced. This genome region spans seven hypervariable regions (HVR) that have been described as the genetic counterpart for antigenic epitopes that contribute to adenovirus serotype characteristics. In particular, HVRs 4, 5 and 7 have been described to be serotype specific [Takeuchi et al., 1999]. For this reason, sequencing of the HVRs has been suggested for adenovirus genotyping [Blasiole et al., 2004; Crawford-Miksza and Schnurr, 1996; Li et al., 1999; Takeuchi et al., 1999].

In the HVR spanning region, the virus investigated showed the highest homology to a partial hexon database sequence of an adenovirus which was detected in a throat wash sample. This virus was classified as adenovirus 11, but no further information on the way of serotype identification or the clinical setting was available. Merely, the site of isolation suggests a clinical state of respiratory disease. Compared to the adenovirus 11 prototype strain (Slobitski), our sequence showed a lower grade of homology, and also in the deduced protein sequence the virus was not identical to this strain. These differences also affected the HVRs (HVR 1, 4, 6 and 7), which queried the classification as an adenovirus 11 genotype.

To elucidate further this question, a part of the fiber gene was examined, since additional type specific epitopes are located on this viral protein [Hierholzer, 1992]. The sequence obtained, spanning more than one half of this gene, showed the

highest homology to a database entry of an adenovirus 11 strain (BC34) that was isolated from a throat swab of a patient with respiratory infection and identified as an adenovirus 11a genome type [Mei and Wadell, 1993]. The homology to the adenovirus 11 Slobitski strain (Adenovirus11p) again was lower. The subdivision of adenovirus 11 into different genome types has been suggested due to the identification of three genomic clusters [Li et al., 1991]. The adenovirus 11a cluster was described to exclusively comprise viruses that were isolated from patients with respiratory disease, while viruses representing the other genome types, like the prototype strain Slobitski (Adenovirus11p), were isolated from healthy pregnant women and renal transplant recipients with infections of the urinary tract [Li et al., 1991]. A total of 25 amino acid changes in the fiber sequences of adenovirus 11a and adenovirus 11p allows a reliable differentiation of these two genomic clusters [Mei and Wadell, 1993]. Since the analyzed virus had a fiber amino acid sequence that was identical to adenovirus 11a, but differed in 16 of 193 amino acids to adenovirus 11p, it could be clearly identified as an adenovirus 11a genotype.

In general, species B2 adenoviruses are isolated very rarely (<1%) from clinical samples [Li et al., 1991], since they cause disease mainly in immunocompromised patients [Hierholzer, 1992; Mei et al., 2003]. This is in accordance with serological studies which found the prevalence of adenovirus 11-specific antibodies to be low [Li et al., 1991; Mei et al., 2003]. To our knowledge, adenovirus 11 has been reported only once as the causative agent of acute respiratory disease in a military facility. In 1974, an atypical adenovirus 11 strain was isolated from Spanish recruits with rather mild clinical symptoms. Indeed, this virus caused only part of the outbreak, since other adenovirus serotypes and influenza virus have also been detected [Hierholzer et al., 1974]. It was identified as adenovirus 11 by serum neutralization, but

surprisingly the virus failed to agglutinate monkey erythrocytes [Hierholzer et al., 1974], which later was described to be characteristic for adenovirus 11a strains [Mei and Wadell, 1993]. Therefore, the Spanish outbreak probably also was caused by the adenovirus 11a genome type.

Although the occurrence of acute respiratory disease due to adenovirus infection of military personnel is a recurring event, a species B2 adenovirus being the causative agent of severe respiratory symptoms is exceptional and underlines the necessity of generic adenovirus diagnosis, even in clinical settings which suggest particular serotypes as the causative pathogen.

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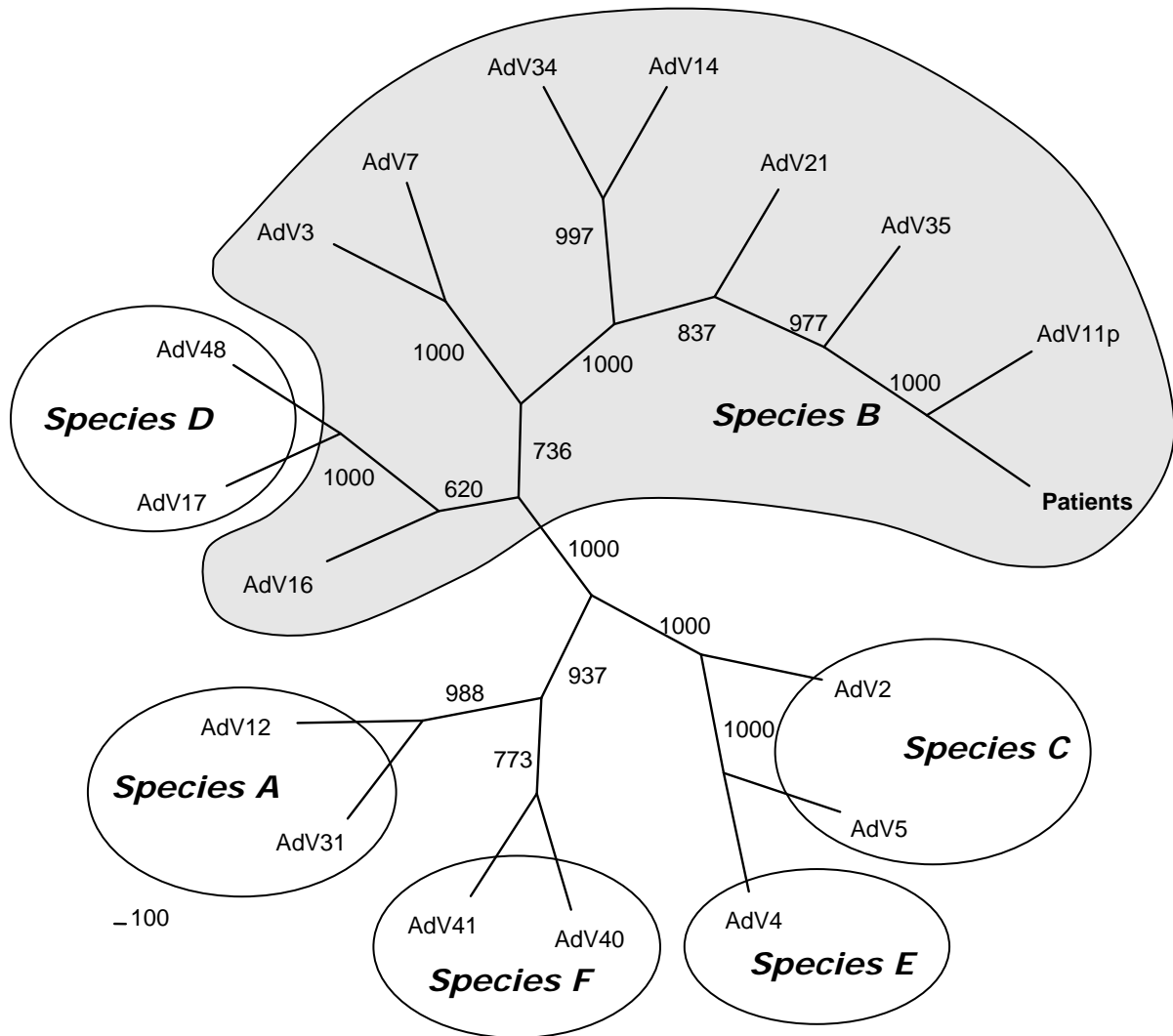
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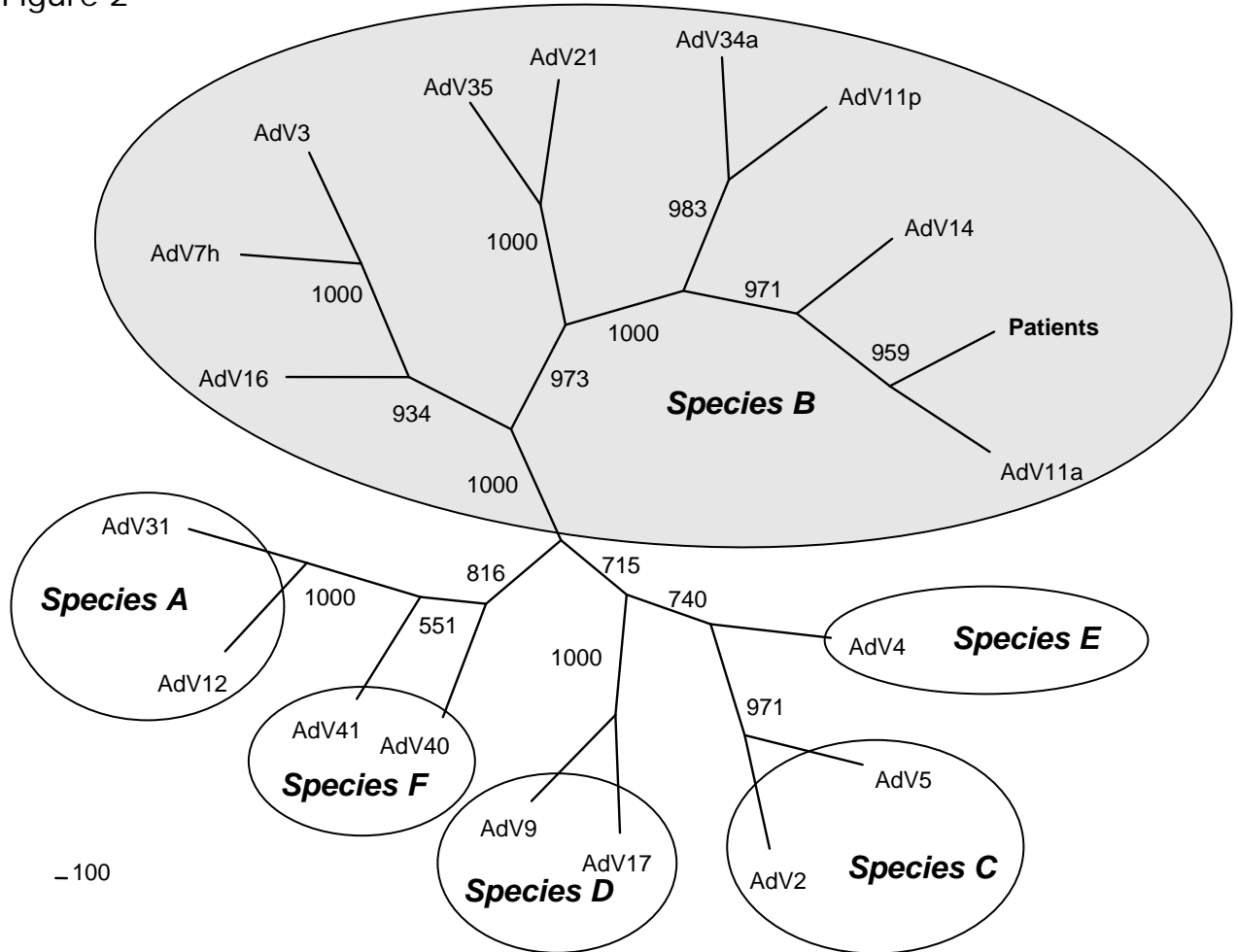Wilhelmi I, Roman E, Sanchez-Fauquier A. 2003. Viruses causing gastroenteritis. Clin Microbiol Infect 9(4):247-262.

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Figure 1

Phylogenetic analysis of the putative protein sequences of the Hexon gene (410aa): AdV11p (AF532578), AdV14 (AB018425), AdV34 (AB018426), AdV35 (AB018427), AdV3 (X76549), AdV7 (AF515814), AdV16 (X74662), AdV21 (AY008279), AdV12 (X73487), AdV31 (X74661), AdV2 (AF542120), AdV5 (AF542128), AdV17 (AF108105), AdV48 (U20821), AdV4 (AF065063), AdV40 (X51782), AdV41 (D13781); the unknown virus can clearly be grouped into species B, showing the highest homology to AdV11p [the sequence of the clinical AdV11 isolate (strain 91-038T, accession number AB162772) was not included, because only little information on typing and derivation was available].

Figure 2

Phylogenetic analysis of the putative protein sequences of the fiber gene (193aa): AdV11a (L08232), AdV11p (AF532578), AdV14 (AB065116), AdV34a (U10271), AdV35 (U32664), AdV3 (AY224416), AdV7h (Z48954), AdV16 (U06106), AdV21 (U06107), AdV12 (X73487), AdV31 (X76548), AdV2 (J01917), AdV5 (M18369), AdV9 (X74659), AdV17 (Y14241), AdV4 (AB098607), AdV40 (M28822), AdV41 (M60327); the unknown virus can clearly be grouped into species B, showing the highest homology to AdV11a.

Table 1   Oligonucleotides

gene	name		oligonucleotide sequence (5' – 3' orientation)	amplicon[§]	T$_a$
fiber [§§]	P-016	s	TSTACCCYTATGAAGATGAAAGC	670-772 bp	54°C
		as	GGATAAGCTGTAGTRCTKGGCAT		
Hexon	P-060	s	GCATACATGCACATCGCCG	2.793 bp	60°C
		as	AGAACGGTGTACGCAGGTAGAC		
Hexon	P-061	s	GACAGGATGCTTCGGAGTACC	2.729 bp	60°C
		as	GCTGATGCACTCTGACCACG		
Hexon	P-062/1	s	GGGGTGTGTTGGACAGAGGTC	Seq	55°C
Hexon	P-062/2	s	CCTAAAATTGTCATGTATGCAGAAAATG	Seq	55°C
Hexon	P-062/3	s	AATTAACCTTCAAGCCAATCTATGG	Seq	55°C
Hexon	P-062/4	s	CTGGGAGATGAAACTTGGACTG	Seq	55°C
Hexon	P-062/1	as	GGCATCCAGAGACCACCTG	Seq	55°C
Hexon	P-062/2	as	GAGACGCTTGACCAGCCAG	Seq	55°C
Hexon	P-062/3	as	CCATCAAGGTCAGTCCAAGTTTC	Seq	55°C
Hexon	P-062/4	as	GTGGTGGTTGAATGGGTTGAC	Seq	55°C

s = sense orientation

as = antisense orientation

T$_a$ = Annealing temperature

Seq = primer for sequencing

§ = length of obtained PCR product

§§ = specific for species B AdV [Xu, McDonough et al. 2000]

Table 2　　Sequence homologies of the patients' virus to prototype sequences (in % identity)

		Adenovirus serotypes of species...											
		B2					B1					C	E
		11a	11p	14	34	35	3	7	16	21	50	2	4
Hexon	DNA	n.d.	**97.1**	82.0	79.5	87.2	69.0	69.1	69.6	81.7	n.d.	59.8	66.7
	AA	n.d.	**96.6**	81.2	80.2	87.0	72.1	73.4	71.8	85.7	n.d.	59.3	59.1
Fiber	DNA	**99.8**	92.9	99.3	92.6	70.6	62.3	62.3	51.7	69.3	n.d.	39.6	34.8
	AA	**100.0**	91.7	98.4	91.2	64.1	55.5	55.5	47.5	61.5	n.d.	<20	20.9

Percent identities were calculated on the basis of sequence alignments (ClustalW) with the DNA Star Software Package (MegAlign); the serotype showing the highest sequence identity (DNA + protein level) is written in bold

DNA = nucleotide level

AA = amino acid level

n.d. = not determined

The database sequences for this analysis were:

Hexon gene:　AdV11p (AF532578), AdV14 (AB018425), AdV34 (AB018426), AdV35 (AB018427), AdV3 (X76549), AdV7 (AF525814), AdV16 (X74662), AdV21 (AY008279), AdV2 (AF542120), AdV4 (AF065063); the sequence of the clinical AdV11 isolate (strain 91-038T, accession number AB162772) was not included, as only little information on typing and derivation was available

Fiber gene:　AdV11a (L08232), AdV11p (AF532578), AdV14 (AB065116), AdV34a (U10271), AdV35 (U32664), AdV3 (AY224416), AdV7h (Z48954), AdV16 (U06106), AdV21 (U06107), AdV2 (J01917), AdV4 (AB098607)