Virchows Arch (1996) 428:113–118

ORIGINAL ARTICLE

T. Bajanowski · P. Wiegand · R. Cecchi
P. Pring-Åkerblom · T. Adrian · G. Jorch
B. Brinkmann

Detection and significance of adenoviruses in cases of sudden infant death

Received: 18 August 1995 / Accepted: 18 December 1995

Abstract Respiratory tract infections have been thought to act as a trigger mechanism in sudden infant death. In 118 autopsy cases of infant death, paraffin-embedded or frozen lung tissues were investigated by means of a nested polymerase chain reaction (PCR) to detect adenovirus (AV) DNA. The primers used are general primers and allow the detection of most pathogenic adenoviruses with high specificity and sensitivity and independently of devitalization of viruses or degradation of viral DNA. For the investigation three groups were established: there were 13 cases of unnatural death, 78 cases of natural death without histological signs of interstitial pneumonia, and 27 cases with interstitial pneumonia. The first group was AV negative. In the group without interstitial pneumonia AV was detected in 10.2% of the cases. In the group with interstitial pneumonia the frequency of AV detection was almost 26%. The results obtained demonstrate an association between interstitial pneumonia and detection of AV DNA, indicating that AV may play an important part in pulmonary infection in infants. Histological evidence of interstitial pneumonia was not observed in all AV-positive cases, perhaps because nonspecific virus-related changes occurred only in early stages of viral infection. Comparison of the AV frequency in SIDS (15%) and non-SIDS cases (4%) indicates an association between pulmonary AV infections and sudden death. These results support the working hypothesis of respiratory infections acting as a trigger mechanism in sudden infant death.

Key words Sudden infant death syndrome · Interstitial pneumonia · Adenovirus detection · Nested PCR

T. Bajanowski · P. Wiegand · R. Cecchi · B. Brinkmann (✉)
Institut für Rechtsmedizin, Westfälische Wilhelms-Universität,
Von Esmarch-Strasse 86, D-48149 Münster, Germany
Tel.: (49) 251–83 51 61; Fax: (49) 251–83 51 58

R. Cecchi
Istituto di Medicina Legale e delle Assicurazioni,
Viale Regina Elena, 336, I-00161 Rome, Italy

P. Pring-Åkerblom · T. Adrian
Nationales Referenzzentrum für Adenoviren,
Institut für Virologie und Seuchenhygiene,
Medizinische Hochschule, Konstanty-Gutschow-Strasse 8,
D-30625 Hannover, Germany

G. Jorch
Klinik und Poliklinik für Kinderheilkunde,
Westfälische Wilhelms-Universität, Albert-Schweitzer-Strasse 33,
D-48149 Münster, Germany

Introduction

Viral infections of the respiratory system and the digestive tract have been considered to act as trigger mechanisms in the sudden infant death syndrome (SIDS) [5, 24, 30]. Although changes associated with viral infections are defined histologically [10, 22, 23], it is difficult to demonstrate a viral origin, because the histological changes are neither virus specific nor attributable to a specific type of virus [10, 22, 23]. The detection of a specific type of virus would verify the diagnosis, and we have therefore developed a nested PCR approach for the detection of adenovirus (AV) DNA and tested its applicability and specificity in autopsy samples.

Adenoviruses were first isolated from resected human adenoids by Rowe et al. [21]. The virus possesses a capsid consisting of 252 capsomers [13]. The double stranded DNA has a weight of 23×10^6 Da and a length of 36 kb [18]. Only some of the 47 known antigenically distinct serotypes affect humans. The most important diseases caused by AV are epidemic keratoconjunctivitis [14], acute gastroenteritis, pharyngitis and diseases of the respiratory tract, including pneumonia [7].

Materials and methods

Subjects

All infant deaths (n=118) in which postmortem examinations had been carried out at the Institute of Forensic Medicine in Münster in the period 1991–1993 were included in this study. The investigation comprised examination of the scene of death, a full autopsy

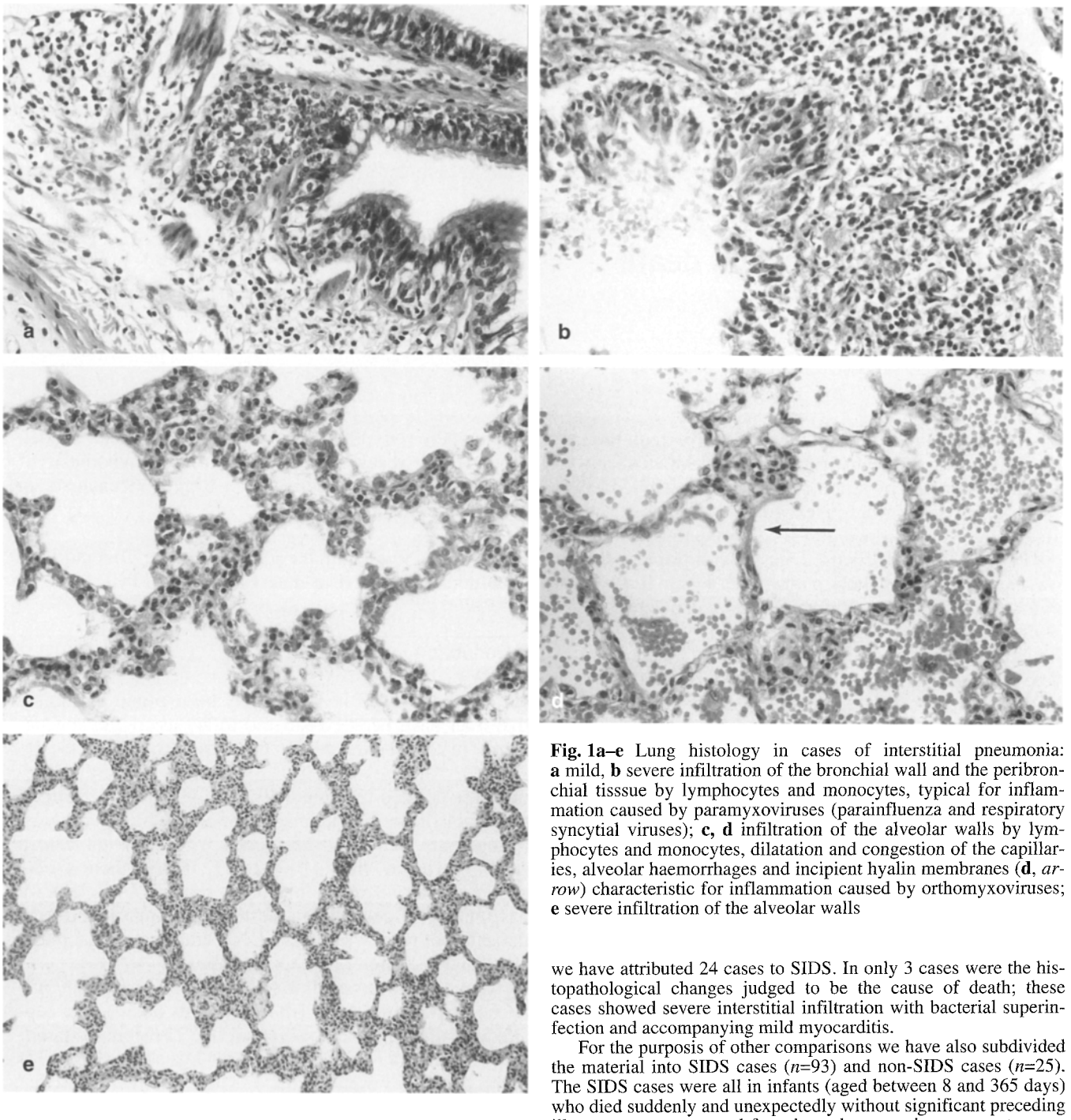

Fig. 1a–e Lung histology in cases of interstitial pneumonia: **a** mild, **b** severe infiltration of the bronchial wall and the peribronchial tisssue by lymphocytes and monocytes, typical for inflammation caused by paramyxoviruses (parainfluenza and respiratory syncytial viruses); **c, d** infiltration of the alveolar walls by lymphocytes and monocytes, dilatation and congestion of the capillaries, alveolar haemorrhages and incipient hyalin membranes (**d**, *arrow*) characteristic for inflammation caused by orthomyxoviruses; **e** severe infiltration of the alveolar walls

using a standard autopsy protocol similar to that of the SIDS Global Strategy Workshop [6], histology on 28 organs and tissues and regular toxicological screening.

Lung histology (samples from all five lobes; haematoxylin and eosin, alcian blue–PAS, semi-thin sections) allowed the definition of a subgroup (n=27) showing different grades of interstitial pneumonia (IP; classification according to Schäfer [22], Entrup and Brinkmann [10]). On the basis of further autopsy findings and histopathology the cases were finally divided into 13 unnatural deaths and 105 natural deaths, which included 93 cases of SIDS and were further subdivided into cases with no morphological signs of virus infection (69 SIDS cases and 9 cases with defined natural cause of death) and 27 cases with signs of virus infection (various grades of IP; see Fig. 1). Because of the results of quantitative expression we have attributed 24 cases to SIDS. In only 3 cases were the histopathological changes judged to be the cause of death; these cases showed severe interstitial infiltration with bacterial superinfection and accompanying mild myocarditis.

For the purposis of other comparisons we have also subdivided the material into SIDS cases (n=93) and non-SIDS cases (n=25). The SIDS cases were all in infants (aged between 8 and 365 days) who died suddenly and unexpectedly without significant preceding illness or symptoms and for whom the extensive postmortem examination failed to demonstrate an adequate cause of death.

Three specimens taken from the three right lung lobes were frozen in liquid nitrogen and stored for up to 24 months at –70°C for the virus examination. DNA extraction (approximately 20 mg tissue/case) was carried out from one randomly selected specimen with proteinase K lysis, phenol/chloroform purification and centricon 100 concentrators (Amicon, Witten, Germany). In 18 cases only paraffin-embedded tissues were available, and 4-µm lung sections were deparaffinized by xylol/ethanol before proteinase K lysis at 37°C overnight. As template DNA for PCR, 1 µl of the final DNA extract (50 µl) was used either undiluted or in a final dilution of 1:10. For further investigation the extracted DNA was marked with a code so that those performing the PCR analysis were blind to the histology and to the cause of death.

Table 1 Causes of death in cases of unnatural and of natural death (*IP* interstitial pneumonia, *SIDS* sudden infant death syndrome)

Unnatural deaths (n=13)		Natural deaths (n=105)	
Head injury	4	*Without IP* (n=78)	
Drowning	3	Sepsis	3
Suffocation	3	Pneumonia due to aspiration	2
Cocaine poisoning	1	Bronchopneumonia	2
Embolism of pulmonary arteries due to injection of carotide juice	1	Exsiccosis	1
		Bronchopulmonary dysplasia	1
		SIDS	69
Laryngeal bolus	1		
		With IP (n=27)	
		Myocarditis	2
		Sepsis	1
		SIDS	24

Table 2 Distribution by sex and by month of death in each group of cases studied

Group	Total	Male	Female		Month of death			
	n	n	n	(%)	Oct.–March		April–Sept.	
					n	(%)	n	(%)
Unnatural deaths	13	10	3	(30)	8	(61)	5	(39)
Natural deaths without IP	78	47	31	(40)	48	(61)	30	(39)
Natural deaths showing IP	27	15	12	(44)	12	(44)	15	(56)
Total	118	72	46	(39)	69	(58)	49	(42)

PCR protocol

For the first amplification the primer pair HA1 and rH1 was evaluated. These primers amplify a 321-bp fragment of the 5′-conserved hexon gene region equivalent to nucleotide positions 1–321 with reference to AV2 and AV5 [1, 15]. The amplification conditions, using 1 µl template DNA, were 1 U Taq-polymerase (Promega, Madison, Wis.), 0.3 µmol each primer, 100 µmol of each dNTP, 2 µl PCR buffer (Promega, Madison, Wis.), diluted to a total volume of 25 µl with distilled water. The reaction mixture was overlaid with 2 drops of oil.

The primer sequences used were HA1 (forward primer) 5′-ATGGCTACCCCTTCGATGAT′ and rH1 (reverse primer) 5′-CAGCACGCCGCGGATGTCAA′.

The first amplification used the following sequence 94°C – 1 min (denaturation), 56°C – 1 min (annealing), 72°C – 1 min (extension) and was repeated for 19 cycles (thermocycler Perkin-Elmer Cetus 9600).

Of 25 µl from the first amplification, 2 µl was used in the nested step. The amplification conditions were identical to those in the first PCR except that 30 cycles were carried out, and the primer sequences used were np1 (forward primer) 5′-AGACGTACTTGAGCCTGAA′ and np2 (reverse primer) 5′-CCTTGTACGAGTACGCAGTA′.

The primer sequences were selected from the sequence structure of AV2 [1, 15] using the program "Primer 0.5" (Whitehead Institute for Biomedical Research). The final PCR product is a 138-bp fragment located in the 5′-conserved hexon region of the hexon gene (nucleotide positions 107–244).

Electrophoresis was carried out using polyacrylamide gels (8%, 0.45 mm thick) with a separation distance of 10 cm. The PCR products were visualized by silver staining [4]. Both positive controls (purified DNA of AV2) and blanks were investigated together with the samples. For fragment size calibration the 123 bp ladder was used (Gibco BRL, Eggenstein, Germany). As a further check on the specificity of the PCR products, fragments obtained in 5 positive cases (3 cases with IP, 2 cases without infection; all 5 SIDS cases) were sequenced using Taq cycle sequencing (ABI sequencer 373a, ABI, Foster City, Calif.) according to Möller et al. [17].

The presence of antibodies against adenovirus antigens was investigated in all cases using the complement fixation reaction.

Results

The causes of death in the controls and in the cases of natural death are shown in Table 1. The sex distribution did not vary much in the subgroups formed (Table 2). The age distribution was quite similar in groups with and without signs of virus infection, while that among the unnatural deaths was associated with clearly higher mean values (Fig. 2). The postmortem intervals (the time between the presumed time of death and autopsy) showed no wide differences with group (Fig. 2). About 60% of the victims died in the autumn and winter months (Table 2), except for those with IP, among whom the proportion is nearly inverse (44% in autumn and winter); this difference is not statistically significant, however (Chi-square test). The incidence of AV-positive cases varied between zero for unnatural deaths to 10.2% in infants with no evidence of infection and 26% in those with IP (Table 3). Of 7 AV-positive cases showing changes, 3 showed bronchiolitis and focal peribronchial infiltration, while the remaining cases were associated with a more diffuse type of lymphomonocytic infiltration of the alveolar walls. The differences between the subgroups with natural death are significant at the 5% level (Chi-square test).

The sex distribution in SIDS and non-SIDS cases and the ratio of victims who died in autumn and winter months is nearly identical in the two groups and also in the entire series (Table 4). Differences in the average age and the postmortem intervals are not significant. The association of AV findings with SIDS and non-SIDS cases revealed incidences of 15% and 4%, respectively (Table 5). These differences are not significant (χ^2=2.17).

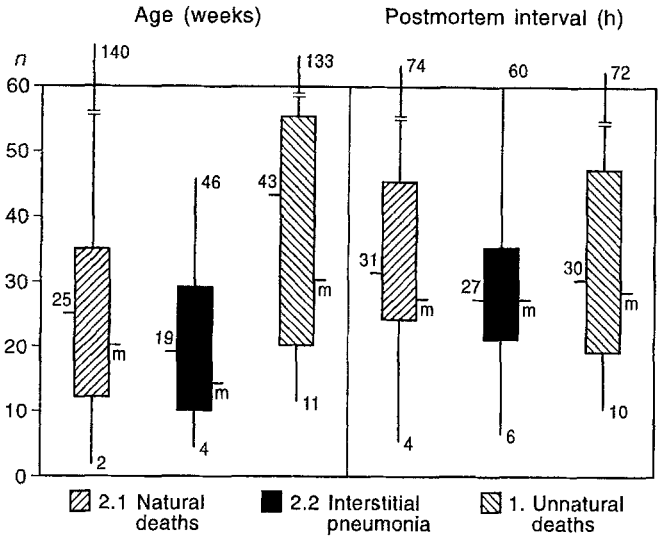

Fig. 2 The box-and-whisker plots of age (weeks) and postmortem interval (h) in the three groups investigated (unnatural death, natural death with and without interstitial pneumonia) are shown. The *"boxes"* show the range between the 25th and 75th percentiles. The *"whiskers"* mark the range between the smallest and highest values, also given numerically. The *horizontal lines* marked with *"m"* give the median of the distribution and the *horizontal lines* marked with *numbers* show the arithmetic mean age and PMI in the groups

Of the 18 cases in which the DNA was extracted from paraffin-embedded material, 2 were positive for AV DNA (11.1%): 1 was a SIDS case without IP; the other was also a SIDS case but with signs of IP. The frequency of AV detection among the cases with DNA extraction from frozen samples was 13%. No differences in the intensity of the fragments attributable to the extraction method were observed (Fig. 3). Sequencing gave an identical DNA sequence in all 5 cases corresponding to the published sequence of AV 2 [1, 15]. Antibodies against AV antigens were only detected in 2 of the AV-positive SIDS cases showing no IP, while the remaining 13 positive cases were negative for antibodies.

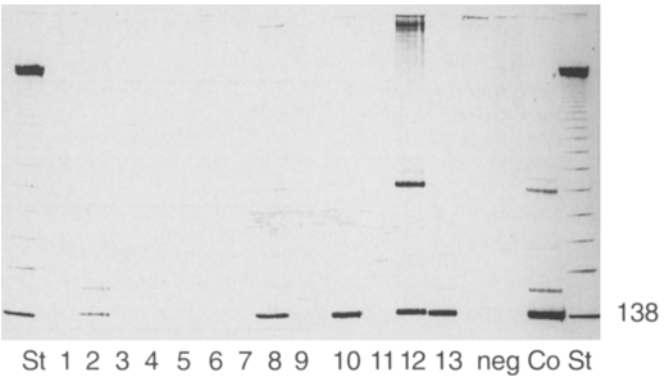

Fig. 3 Detection of adenovirus DNA using nested PCR followed by polyacrylamide gel electrophoresis and silver staining. On the first and last position (*St*) the 123-bp ladder (Gibco BRL) is shown as fragment size control. The 138-bp fragment in *lanes 2, 8, 10, 12* and *13* indicates the presence of viral DNA (also sequenced). The fragment in *lane 2* is weak. Positive (*Co*) and negative (*neg*) controls are also included. The template DNA used in the case shown in *lane 8* was obtained from paraffin-embedded tissue. In the other cases, DNA extraction was carried out using frozen material

Table 3 Adenovirus (*AV*) detection in each group of cases studied

Group	Number of cases (*n*)	AV-positive cases (*n*)	Proportion of cases positive (%)
Unnatural deaths	13	0	0*
Natural deaths without IP	78	8[a]	10.2*
Natural deaths with IP	27	7[b]	25.9
Total	118	15	12.7

* P=0.05 (difference between group with IP and the other groups; Chi-square test)
[a] Two AV-positive cases without IP were sequenced
[b] Three AV-positive cases with IP were sequenced

Table 4 Distribution by sex and by month of death in SIDS and non-SIDS cases

Group	Total (*n*)	Male (*n*)	Female (*n*)	(%)	Month of death			
					Oct.- March (*n*)	(%)	April-Sept. (*n*)	(%)
SIDS	93	56	37	(40)	54	(58)	39	(42)
Non-SIDS	25	16	9	(36)	15	(60)	10	(40)
Total	118	72	46	(39)	69	(58)	49	(42)

Table 5 AV detection in SIDS and non-SIDS cases

Group	Number of cases (*n*)	AV-positive cases (*n*)	Proportion of cases positive (%)
SIDS	93	14[a]	15.0
Non-SIDS	25	1	4.0
Total	118	15	12.7

[a] In 5 of the AV-positive SIDS cases the amplified fragment was sequenced

Discussion

Coumbe et al. [8] investigated tissue samples from 40 SIDS cases using in situ hybridization. They found no evidence to suggest that cytomegalovirus or human herpesvirus 6 is involved in causing SIDS by way of overwhelming viraemia. The results of An et al. [3] indicate that AV could be the most frequent cause of respiratory viral infections in infants. The most common serotypes causing respiratory diseases are AV 1, 2, 5, 6 (all subgenus C) and occasionally AV 3 (subgenus B) [27]. The histopathology of AV pneumonia was first described by Goodpasture et al. [11] and is characterized by necrotizing bronchitis and bronchiolitis with desquamation of epithelium [22, 25], haemorrhages and areas of consolidation and atelectasis. The cytopathic effects of AV are expressed in the form of intranuclear inclusion bodies [22].

The present methods of virus detection (culture and electron microscopy, immunofluorescence and serology) may give false-negative results or not provide evidence of active viral infection [3]. The postmortem interval (in our cases an average of 29 h) is regularly associated with degradation and devitalization of viruses. Several AV are fastidious and difficult to grow in culture [9, 12, 28]. The limit of resolution for electron microscopy (108 particles per ml; Gelderblom, personal communication, cited in [20]) is relatively high. At present, PCR is the most sensitive method of virus detection [2] and is not dependent on virus devitalization.

The results of the present study provide further evidence for the viral aetiology of IP in some cases. It was also demonstrated that AV infection is more frequent in SIDS (15.0%) than in cases of unnatural death (0%). As AV3 does not belong to subgenus C, the 5′-conserved hexon gene region might differ to such an extent that it cannot be amplified with the primers selected from the AV2 nucleotide sequence. This suggests that AV infection might be more frequent than has been detected in this series. Furthermore, AV infection is a frequent and therefore important cause (25.9%) of IP. The method applied has a high sensitivity, and the specificity was confirmed by Taq cycle sequencing. The primers HA1 and rH1 do not allow differentiation between AV subtypes of subgenus C, because the homology of the DNA sequence of the amplified fragment of AV 2 and AV 5 is 100% [19].

An et al. [3] investigated lung tissue by means of in situ hybridization. The virus detection was successful in 24% of the SIDS cases (n=45) and in 3% of the non-SIDS cases (n=30), and in 72% of the positive cases the viruses found were AV. The AV incidence in their SIDS cases was 17.7%, similar to the incidence in the present study (15.0%).

It should be mentioned that 3 additional positive cases were found, in which the results of nested PCR were not reproducible. These cases were not recorded as AV positive. It can only be supposed that the amount of template DNA in these cases was near the detection limit.

In 8 of 15 AV-positive cases (53.3%), no signs of interstitial inflammation were found histologically. There are three possible explanations for this. (1) The first stage of IP is characterized only by nonspecific findings (haemorrhages, oedema or desquamation of epithelial cells [10]), and that such changes may be caused by virus infection, but not exclusively. It is therefore impossible to diagnose this early stage of IP specifically by histology. (2) AV detection in some cases without IP may result from viraemia caused by AV infection from another location (for example, the gut). At present it is not clear whether the primers used are able to detect AV 40 and 41, which are involved in the aetiology of acute gastroenteritis (P. Pring-Åckerblom 1995, personal communication). The persistence of AV in lymphocytes of normal human hosts has been documented for 24 months after initial infection [16]. (3) Finally, in the 2 cases in which antibodies against AV were found but no IP, a reactivation of adenovirus infection may have occurred.

AV detection was successful regardless of whether or not the formalin-fixed specimens were used (success rate in formalin fixed specimerns: 11%; success rate in frozen specimens: 13%). This indicates the suitability of the method developed for DNA detection in autopsy samples. In only 13% (n=2) of the AV-positive cases were the victims younger than 3 months at death. This does not contradict the findings reported by An et al. (37.5% [3]) for this age group, because the differences are not significant (Fisher test, P=0.05). Our results are also consistent with those of Uren et al. [26] and Williams et al. [29], who reported a substantially higher viral infection rate in infants older than 3 months.

References

1. Akusjärvi G, Aleström P, Pettersson M, Lager M, Jörnvall H, Pettersson U (1984) The gene for the adenovirus 2 hexon polypeptide. J Biol Chem 259: 13976–13979
2. Allard A, Girones R, Juto P, Wadell G (1990) Polymerase chain reaction for the detection of adenoviruses in stool samples. J Clin Microbiol 8: 2659–2667
3. An SF, Gould S, Keeling JW, Fleming KA (1993) Role of respiratory viral infection in SIDS: detection of viral nucleic acid by in situ hybridization. J Pathol (Lond) 171: 271–278
4. Bajanowski T, Wiegand P, Brinkmann B (1994) Comparison of different methods for CMV detection. Int J Legal Med 106: 219–222
5. Blackwell CC, Saadi AT, Raza MW, Weir DM, Busuttil A (1993) The potential role of bacterial toxins in Sudden Infant Death Syndrome (SIDS). Int J Legal Med 105: 333–338
6. Brooks DR, Krous HF, Burton JL, McKinley J, McKinley W (1994) Results of a pilot test of the global strategy workshop's international standardized autopsy protocol (SAP) in Georgia. In: Sudden infant death syndrome (SIDS), Third SIDS International Conference, Stavanger, Norway, 31.07.1994–04.08.1994, program and abstracts, p 155
7. Costa J, Rabson W (1985) Viral diseases. In: Kissane JM (ed) Anderson's pathology. Mosby, St Louis Toronto Princeton, pp 358–360
8. Coumbe A, Fox JD, Briggs M, Tedder RS, Berry CL (1990) Cytomegalovirus and human herpesvirus-6 in sudden infant death syndrome: an in-situ hybridization study. Pediatr Pathol 10: 483–490
9. DeJong JC, Wigand R, Kidd AH, Wadell G, Kapsenberg JG, Muzerie CJ, Wermenbol AG, Firtzlaff RG (1983) Candidate adenovirus 40 and 41: fastidious adenoviruses from human infant stool. J Med Virol 11: 215–231

10. Entrup M, Brinkmann B (1990) Histologische Lungenbefunde beim plötzlichen Kindstod. Z Rechtsmed 103: 425–433
11. Goodpasture EW, Auerbach SH, Swanson HS, Cotter EF (1939) Virus pneumonia of infants secondary to epidemic infections. Am J Dis Child 57: 997–1011
12. Hanna L, Jawetz E (1962) Attempts to enhance infectivity of adenovirus type 8 for cell culture. Proc Soc Exp Biol 110: 707–709
13. Horne RW, Brenner S, Waterson AP, Wildy P (1959) The icosahedral form of an adenovirus. J Mol Biol 1: 84–86
14. Jawetz E, Kimura S, Nicholas AN, Thygeson P, Hanna L (1955) New type of APC virus from keratoconjunctivitis. Science 122: 1190–1192
15. Kinloch R, Mackay N, Mautner V (1984) Adenovirus hexon. Sequence comparison of subgroup C serotypes 2 and 5. J Biol Chem 259: 6431–6436
16. Knipe DM (1990) Adenoviruses. In: Fields BN (ed) Virology. Raven Press, New York, p 1729
17. Möller A, Meyer E, Brinkmann B (1994) Different types of structural variation in STRs: HumFES/FPS, HumVWA and HUMD21S11. Int J Legal Med 106: 319–323
18. Philipson L (1983) Structure and assembly of adenoviruses. In: Doerfler W (ed) The molecular biology of adenoviruses. Springer, Berlin Heidelberg New York, pp 1–52
19. Pring-Åckerblom P, Adrian T (1993) The hexon genes of adenoviruses of subgenus C: comparison of the variable regions. Res Virol 144: 117–127
20. Pring-Åkerblom P, Adrian T (1994) Type and group-specific polymerase chain reaction for adenovirus detection. Res Virol 145: 25–35
21. Rowe WP, Huebner RJ, Gilmore LK, Parrott RH, Ward TG (1953) Isolation of a cyto-pathogenic agent from human adenoids undergoing spontaneous degeneration in tissue culture. Proc Soc Exp Biol Med 84: 570–573
22. Schäfer HE (1983) Inflammatory diseases of the human lung of definite or presumed viral origin. Cytologic and histologic topics. In: Müller (ed) Pulmonary diseases. Springer, Berlin Heidelberg New York, pp 153–205
23. Spencer H (1985) Pneumonias due to rickettsiae, chlamydiae, viruses and mycoplasma. In: Spencer H (ed) Pathology of the lung. Pergamon Press, Oxford New York, pp 213–259
24. Stoltenberg L, Saugstad OD, Rognum TO (1992) Sudden infant death syndrome victims show local immunoglobulin M response in tracheal and immunoglobulin A response in duodenal mucosa. Pediatr Res 31: 372–375
25. Strano AJ, Henson DE (1975) Fatal adenovirus pneumonia: a study of 17 cases (abstract). Lab Invest 31: 346
26. Uren EC, Williams AL, Jack I, Rees JW (1980) Association of respiratory virus infection with sudden infant death syndrome. Med J Aust 1: 417–419
27. Wadell G (1987) Adenoviruses. In: Zuckerman AJ, Banatvala JE, Patterson JR (eds) Principles and practise of clinical virology. Wiley, Chichester New York, pp 251–274
28. Wigand R, Gelderblom H, Özel M, Distler H, Adrian T (1983) Characteristics of mastadenovirus h8, the causative agent of epidemic keratoconjunctivitis. Arch Virol 76: 307–319
29. Williams AL, Uren EC, Bretherton L (1984) Respiratory viruses and sudden infant death. BMJ 288: 1491–1493
30. Wilske J (1993) Zur Definition und Pathophysiologie des plötzlichen Säuglingstodes. In: Kruse K, Oehmichen M (eds) Plötzlicher Säuglingstod. (Themen der Kinderheilkunde, vol 6) Hansisches Verlagskontor, Lübeck, pp 7–18