Int J Legal Med (2003) 117 : 237–240
DOI 10.1007/s00414-003-0367-6

SHORT COMMUNICATION

T. Bajanowski · B. Rolf · G. Jorch · B. Brinkmann

Detection of RNA viruses in sudden infant death (SID)

Received: 4 December 2002 / Accepted: 18 February 2003 / Published online: 14 May 2003

Abstract Continuing previous work, reverse transcriptase polymerase chain reaction approaches for the detection of RNA viruses (influenza A+B, parainfluenza virus 3, respiratory syncytial virus) were developed. A total of 118 infant deaths, including 13 cases of non-natural death (nND), 78 cases of natural death (ND) without morphological signs of interstitial pneumonia (IP), 27 cases showing IP (ND+IP), were investigated using frozen lung tissue (*N*= 100) and paraffin-embedded material (*N*=18). In five of the autopsy cases (ND+IP) the influenza B virus genome could be detected and the other types of viruses were completely negative. Together with previous results (detection of adenoviruses and cytomegaloviruses) in the same groups, the frequency of virus detection in the cases with IP was 48% compared with 14% in the ND without IP and 7% in the nND. Significant differences in the frequency of virus detection were also obtained when the cases were divided in SIDS and non-SIDS. The results obtained indicate an association between IP and some viruses and support the hypothesis that respiratory virus infections could act as trigger in sudden infant death.

Keywords Sudden infant death · Interstitial pneumonia · RNA viruses · Reverse-transcriptase polymerase chain reaction

T. Bajanowski · B. Brinkmann (✉)
Institut für Rechtsmedizin, Universitätsklinikum Münster,
Röntgenstrasse 23, 48149 Münster, Germany
Tel.: +49-251-8355161, Fax: +49-251-8355158,
e-mail: brinkma@uni-muenster.de

B. Rolf
Institut für Rechtsmedizin,
Ludwig-Maximilians-Universität München,
Frauenlobstrasse 7a, 80046 München, Germany

G. Jorch
Klinik für Allgemeine Pädiatrie und Neonatologie,
Universitätsklinikum Magdeburg,
Wiener Strasse, 39112 Magdeburg, Germany

Introduction

It has been assumed that infectious diseases can act as triggers in the Sudden Infant Death Syndrome (SIDS) [1, 2, 3]. However, it can become difficult to unequivocally diagnose the underlying disease especially if incipient stages and/or general infections are involved and the same applies to the clear demonstration of the causal bacterial infection. Interstitial pneumonia (IP) as caused by viruses is defined histologically [4, 5] but as early stages can be non-specific [6] detection of the virus would therefore verify the diagnosis. Because virus isolation is rarely positive in autopsy samples we have developed nested PCR approaches to detect different RNA viruses (influenza A and B viruses, parainfluenza virus, respiratory syncytial virus: RSV). These approaches have been applied to autopsy samples from SIDS cases and controls.

Materials and methods

Cases

A total of 105 subsequent cases of sudden infant death (natural deaths ND) and 13 non-natural deaths (nND) were investigated by full autopsy using a standard autopsy protocol [7, 8, 9] which included comprehensive histology of 28 organs and tissues and a broad toxicological screening [8, 9, 10].

Histology of the lungs (all 5 lobes, H&E, alcian blue-PAS, semi-thin sections) revealed a subgroup (*N*=27) showing different grades of IP [6, 8]. The remaining 78 cases of ND were without pulmonary disease.

Frozen lung specimens existed in 100 cases while in 18 cases only paraffin-embedded tissues (4% buffered formaldehyde, fixation time <24h) were available. Three specimens taken from the right lung lobes were stored at –80°C for up to 48 months before the investigation was performed.

Virus detection

Viral RNA was extracted using the RNeasy total RNA kit (Qiagen, Hilden, Germany), eluted from the spin column in 30 µl DEPC-treated water and 11.5 µl of the eluate was used for the first strand cDNA-synthesis (cDNA Cycle Kit, Invitrogen, De Schelp, NL).

Table 1 PCR parameters used for the detection of the different types of viruses

	Primer sequences	$MgCl_2$ concentration (mM)	Cycle parameters		Sensitivity[b]
			°C/s	Cycles	
Influenza A [11]	p 1: CCGTCAGGCCCCCTCAAAGC	1.5	1. PCR		10
	p 2: GACCAGCACTGGAGCTAGGA				
	p 3: CAGAGACTTGAAGATGTCTT	1.5	94/60		
	p 4: TGCTGGGAGTCAGCAATCTG		52/60	30	
Influenza B [11]	p 1: TGTCGCTGTTTGAGACACA	1.5	72/180		1
	p 2: AGTTTTACTTGCATTGAATA				
	p 3: GAAGGCAAAGCAGAACTAGC	1.5			
	p 4: TGGCCTTCTGCTATTTCAAA		2. PCR		
Para-Influenza [12]	p 1: CTCGAGGTTGTCAGGATATAG	2.5	94/30		10
	p 2: CTTTGGGAGTGGAACACAGTT		52/60	31	
	p 3: AGGGCAAGTATTACAGATA	2.5	72/90		
	p 4: ATCTGTATTTAGGAGTGCT				
RSV[a]	p 1: GGTGTTGATCTGCAATCGCCA	3.75	94/60, 56/60, 72/90	each 31 cycles	100
	p 2: AACTTTTTCTGATCATTTGT				
	p 3: AAGTGCTCTACTATCCACA	2.25	94/30, 55/30, 72/60	each 35 cycles	
	p 4: CACTAAATTCCCTGGTAATC				

[a]The primer sequences were obtained according to the sequence of the fusion protein (F) (GenBank U31559).
[b]The sensitivity is given as number of cDNA template molecules.

Nested PCR was carried out in a final volume of 25 µl and from the cDNA synthesis 1 µl was used as template in the first and second rounds of nested PCR. The amplification protocols were optimised in a stepwise fashion for the detection of the different viruses (Table 1). To the primers (1 µM) 0.25 U of Taq polymerase was added, as well as 200 µM of each dNTP.

Samples including positive and negative controls (positive: cDNA samples from virus infected cell cultures; negative: samples without template) were analysed in 8% acrylamide gels and visualised by silver staining [11, 12]. For the sensitivity study, the product of the first PCR was quantified by UVB spectrometry, then serially diluted 10-fold down to 1 molecule in 25 µl. These dilutions were reamplified as aforementioned. The fragments obtained in the positive cases were sequenced by Taq cycle sequencing on an ABI DNA sequencer 373A (ABI, Foster City, CA) [13].

The primer annealing sites for influenza A and B are located in the highly conserved M1 matrix protein (general primer)[14], those for parainfluenza 3 are in the conserved region of the haemagglutinin-neuraminidase-(HN)-gene [15], and primers for the detection of RS-virus were designed to anneal in the fusion protein gene (GenBank U31559).

Results

The methodology developed was specific and sensitive, sequence analysis in all positive cases resulted in cDNA sequences corresponding to the literature [14]. A minimum of 1–100 templates cDNA could be detected after the second PCR (Table 2). The detection of virus genomes using paraffin-embedded material from SIDS cases (N=18) was negative in all instances.

Virus detection was positive for influenza B in 5 cases of ND+IP (18.5%, Fig. 1) but negative in the other groups and for all other viruses (Table 2). In four out of these five cases the IP (grade 1: N=2, grade 2: N=2 [6, 8]) was characterised by typical morphology: dilatation and congestion of alveolar capillaries, protein-rich oedema, necrosis of the alveolar epithelium, denudation of the tracheobronchial mucous membrane, detachment of pneumonocytes, alveolar haemorrhages, hyalin membranes, and

Table 2 Results of the influenza B virus detection (column 2). Additionally previous results of the DNA virus detection in the same cases are given [7, 8].

Groups total number N=118		Influenza B positive		AV positive [8]		CMV positive [7]		Total number in each group	
		N	%	N	%	N	%	N	%
NND	N=13	0		0		1	7.7	1	7.7
ND + IP	N=27	5	8.5	7	26.0	3[a]	11.1	13[a]	48.1
ND, no IP	N=78	0		8	10.3	3	3.8	11	14.1
SIDS	N=93	5	5.4	14	15.0	6[a]	6.4	23[a]	24.7
Non-SIDS	N=25	0		1	4.0	1	4.0	2	8.0

[a]In two out of the three cases a combined viral infection has to be assumed (CMV + AV).

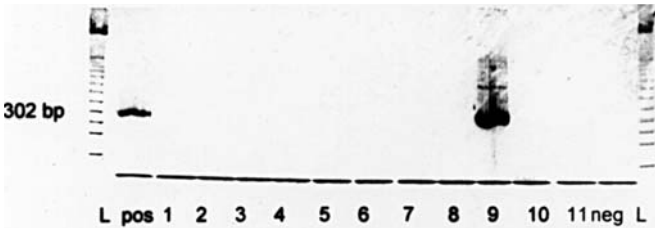

Fig. 1 Detection of influenza B virus. The 302 bp fragment in *lane* 9 indicates the presence of influenza cDNA. Positive (*pos*) and negative (*neg*) controls are included. The 123 bp ladder (Gibco BRL) is used as fragment size control (*L*)

lymphomonocyte infiltration of the alveolar septa [4, 5]. In the fifth case peribronchiolitis was found separately.

Discussion

Coumbe et al. [16] investigated tissue samples from 40 SIDS cases using in situ hybridization for cytomegalovirus (CMV) and human herpesvirus-6 and obtained only negative results. The results of An et al. [17] and our own group [8] indicate that adenoviruses (AV) could be the most frequent cause of respiratory viral infections in infants. Other investigations led to the conclusion that viral myocarditis and enteritis are more common in SID than assumed [18, 19, 20, 21].

In the present study influenza B was associated with IP in all five cases. The atypical peribronchiolar type of IP in one case could indicate an early stage of inflammation [22].

The negative results in the group with preceding formalin fixation of samples can have two reasons: (1) negative for the viruses investigated, (2) negative due to RNA degradation caused by the fixation. Because of lacking adequate controls to resolve this question, this group has been left out of further statistical considerations. In conclusion influenza B infection was associated with SIDS in 6.6% (5 out of 75, 0% in non-SIDS, $p<0.001$).

If the results of DNA virus detection as previously reported [7, 8] are added, viruses causing IP were detectable in 48% (Table2). Moreover, the investigation was successful in 25% of the SIDS cases compared to 8% of the non-SIDS cases. The totally negative results for the detection of influenza A virus, parainfluenza virus 3 and RSV can be due to epidemiological reasons. An especially short postmortem period cannot be made responsible for the positive results because this period was on average 32 h in the whole group as compared to 30 h in the positive cases.

The results of the present study provide further evidence for the viral aetiology of IP in some SIDS cases. IP caused by AV and influenza B does not seem to be a rare disease in infants dying suddenly and unexpectedly and therefore these infections have to be considered at least as a trigger for the death in SIDS.

References

1. 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
2. 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
3. Wilske J (1993) Zur Definition und Pathophysiologie des plötzlichen Säuglingstodes. In: Kruse K, Oehmichen M (eds) Plötzlicher Säuglingstod. Themen der Kinderheilkunde, Band 6. Hansisches Verlagskontor, Lübeck, pp 7–18
4. Schäfer HE (1983) Inflammatory diseases of the human lung of definite or presumed viral origin. Cytologic and histologic topics. In: Müller H (ed) Pulmonary diseases. Springer, Berlin Heidelberg New York, pp 153–205
5. 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
6. Bajanowski T, Brinkmann B (1995) Pulmonary viral infection in SIDS. In: Rognum TO (ed) Sudden infant death syndrome. New trends in the nineties. Scandinavian University Press, Oslo Copenhagen Stockholm Boston, pp 199–202
7. Cecchi R, Bajanowski T, Kahl B, Wiegand P (1995) CMV-DNA detection in parenchymatous organs in cases of SIDS. Int J Legal Med 107:291–295
8. Bajanowski T, Wiegand P, Cecchi R, Pring-Åkerblom P, Adrian T, Jorch G, Brinkmann B (1996) Detection and significance of adenoviruses in cases of sudden infant death. Virchows Arch 428:113–118
9. 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. Sudden infant death syndrome (SIDS). Third SIDS international conference, Stavanger, Norway, 31.07.-04.08.1994, program and abstracts, p 155
10. Bajanowski T, Fürst P, Wilmers K, Beike J, Köhler H, Brinkmann B (2001) Dioxin in infants – an environmental hazard? Int J Legal Med 116:27–32
11. Zhang WD, Evans DH (1991) Detection and identification of human influenza viruses by the polymerase chain reaction. J Virol Methods 33:165–189
12. Karron RA, Froehlich JL, Bobo L, Belshe RB, Yolken RH (1994) Rapid detection of parainfluenza type 3 RNA in respiratory specimens: use of reverse transcription-PCR-enzyme immunoassay. J Clin Microbiol 32:484–488
13. Allen CA, Graves G, Budowle B (1989) Polymerase chain reaction amplification products separated on rehydratable polyacrylamide gels and stained with silver. Biotechniques 7:736–744
14. Budowle B, Chakraborty R, Giusti AM, Eisenberg AJ, Allen RC (1991) Analysis of the variable number of tandem repeat locus D1S80 by the polymerase chain reaction followed by high resolution polyacrylamide gel electrophoresis. Am J Hum Genet 48:137–144
15. 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
16. 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
17. An SF, Gould S, Keeling JW, Fleming KA (1993) Role of respiratory viral infection in SIDS: detection of viral nucleic acid by in situ hybridisation. J Pathol 171:271–278
18. Yolken R, Murphy M (1982) Sudden infant death syndrome associated with rotavirus infection. J Med Virol 10:291–296
19. Shimizu C, Rambaud C, Cheron G et al. (1995) Molecular identification of viruses in sudden infant death associated with myocarditis and pericarditis. Pediatr Infect Dis J 14:584–588

20. Grangeot-Keros L, Broyer M, Briand E et al. (1996) Enterovirus in sudden unexpected death in infants. Pediatr Infect Dis J 15:123–128
21. Dettmeyer R, Kandolf R, Schmidt P, Schlamann M, Madea B (2001) Lympho-monocytic enteroviral myocarditis: traditional, immunohistochemical and molecular pathological methods for diagnosis in a case of suspected sudden infant death syndrome (SIDS). Forensic Sci Int 119:141–144
22. Nishimura T, Fujiyoshi Y, Irie K, Tanimura A (1975) Electron microscopic observations of influenza pneumonitis. Kurume Med J 32:263–271