Int J Legal Med (1995) 107:291–295

ORIGINAL ARTICLE

Rosanna Cecchi · Thomas Bajanowski · Barbara Kahl
Peter Wiegand

CMV-DNA detection in parenchymatous organs in cases of SIDS

Received: 6 March 1995 / Accepted: 20 March 1995

Abstract A nested PCR approach has been developed especially for the detection of small amounts of cytomegalovirus (CMV) DNA in autopsy samples. Lung tissue and submandibular glands in 118 cases of infant death (92 SIDS cases, 13 natural deaths due to other defined causes and 13 unnatural deaths) were investigated by this technique and compared to the results obtained by other CMV detection methods (histology, immunohistochemistry, in situ hybridization and PCR). CMV-DNA could be detected in the lung tissue in 7 cases of SIDS using nested PCR. Compared to conventional PCR (3 positive cases in lung tissue) the nested approach always gave glear results and showed less additional bands. In all cases where CMV could be detected in the lungs, positive results were also obtained in the submandibular glands. The nested PCR method proved to be a more sensitive technique than the other detection methods including PCR and hot start, and even minimal amounts of target DNA could be detected in the presence of human and bacterial background DNA.

Key words CMV inclusion disease · Lung tissue · PCR · Nested PCR

Zusammenfassung Es wurde eine nested PCR Methode speziell für den Nachweis geringer Mengen von Zytomegalievirus DNA in Autopsiematerial entwickelt. In 118 plötzlichen Todesfällen von Säuglingen und Kleinkindern (92 SIDS-Fälle, 13 natürliche Todesfälle anderer definierter Ursache, 13 nichtnatürliche Todesfälle) wurde Lungengewebe und Gewebe der Glandula submandibularis mittels dieser Technik untersucht und mit den Ergebnissen herkömmlicher Methoden (Histologie, Immunhistochemie, in situ Hybridisierung, PCR) verglichen. CMV-DNA konnte dabei durch nested PCR in 7 Fällen im Lungenparenchym nachgewiesen werden. Die "konventionelle" PCR (einschließlich hot start) ergab in 3 Fällen teils schwach positive Ergebnisse. Beim Vergleich beider Methoden zeigte die nested PCR klarere Ergebnisse und weniger Zusatzbanden. In allen Fällen, in denen in der Lunge positive Ergebnisse erzielt wurden, konnte auch in den Speicheldrüsen CMV-DNA nachgewiesen werden. Die nested PCR ist demzufolge verglichen mit PCR und hot start die sensitivste der Methoden und gerade zum Nachweis geringer Mengen von target DNA in Gegenwart von humaner und bakterieller background DNA geeignet. Die Methode kann sowohl für formalinfixiertes, paraffineingebettetes wie auch für tiefgefrorenes Material angewendet werden. Sie ist auch zum Virusnachweis in anderen parenchymatösen Organen (Leber, Nieren) geeignet.

Schlüsselwörter Zytomegalieinfektion · Lungengewebe · PCR · Nested PCR

Introduction

The cytomegalovirus (CMV) inclusion disease is an ubiquitious infection giving rise to typical changes of the ductal cells of salivary glands (Costa and Rabson 1985). The diagnosis of the CMV inclusion disease can be established by histology, immunohistochemistry (IHC), in situ hybridization (ISH) or PCR (Höfler et al. 1988, Wu et al. 1989, Jiwa et al. 1989, Cassol et al. 1989). The detection of CMV in other organs is also possible but the morphological changes, e.g. in lungs, are sometimes minimal and nonspecific. A PCR assay which had been previously applied to CMV detection in salivary glands was found to be no more sensitive than other detection methods (Bajanowski et al. 1994). The aim of the present study was therefore to develop an alternative nested PCR technique

R. Cecchi · T. Bajanowski (✉) · P. Wiegand
Institute of Legal Medicine, Westfälische Wilhelms-Universität, Von-Esmarch-Strasse 86, D-48149 Münster, Germany

B. Kahl
Institute of Microbiology, Westfälische Wilhelms-Universität, Domagkstrasse 10, D-48149 Münster, Germany

R. Cecchi
Institute of Legal Medicine, University of Rome, Viale Regina Elena 336, I-00161 Rome, Italy

and to check the suitability and sensitivity in formalin fixed tissue as well as in frozen samples compared to other CMV detection methods.

Material and methods

The study was carried out on 118 cases of infant death comprising 92 SIDS cases, 13 unnatural deaths and 13 cases due to defined diseases. Specimens from the lung, the submandibular glands, the liver and the kidneys were fixed in 4% buffered formalin and 4 µm sections were stained with H & E for microscopy. Lung sections were investigated in all cases using IHC and ISH and in 77 cases the submandibular glands were also investigated. IHC was carried out employing two monoclonal antibodies, CCH2 and DDG9/CCH2 (DAKO A/S, Glostrup, Denmark). For ISH a DNA Probe Assay for the detection of CMV (ENZO Diagnostics, Farmingdale, N.Y.) was used (Bajanowski et al. 1994). The submandibular glands and the lung tissue in all cases were investigated using PCR and nested PCR. Liver and kidney specimens from cases showing positive reactions in the submandibular glands were further investigated by nested-PCR.

PCR

The method utilized the amplification of a 139 bp fragment in the late antigen GP 64 region which has been described elsewhere (Bajanowski et al. 1994).

Nested-PCR

DNA was extracted from either frozen lung tissue (103 cases: proteinase K lysis, phenol purification and CENTRICON 100 concentrators; AMICON, Witten, Germany) or paraffin embedded tissue (15 cases) – DNA from submandibular glands and kidney and liver specimens were extracted from paraffin embedded tissues (Bajanowski et al. 1994). The phenolized samples were diluted with distilled water to a final volume of 2 ml and centrifuged at 3500 rpm (4° C for 30 min). This procedure was repeated once. The final volume obtained was approximately 50 µl. For the nested PCR a 435 bp fragment encoded in the region of the major immediate early antigen (MIE) was amplified (Wolfe et al. 1990). Two assays were run in parallel: one with 1 µl undiluted extract and one with 1 µl of a 1:50 dilution of the extract. Reaction mixtures were prepared using 0.3 U Taq Polymerase (Goldstar, Eurogentec, Belgium), 0.2 µ*M* of each primer, 100 µ*M* dNTPs, 1.5 µ*M* $MgCl_2$, 2 µl PCR buffer (10 m*M*) (Goldstar) to a final volume of 24 µl, in distilled water. Finally 1 µl of extracted DNA was added and the reaction mixture was overlayed with two drops of oil.

Primer sequences (Wolfe et al. 1990)
CMV-MIEA 1: 5′-CCAAGCGGCCTCTGATAACCAAGCC-3′
CMV-MIEA 2: 5′-CAGCACCATCCTCCTCTTCCTCTGG-3′
Amplification (30 cycles): 94° C – 1 min, 64° C – 1 min, 72° C – 1.5 min.

In the second step a 110 bp fragment was selected. The internal primer pair did not overlap with the external one. This second reaction, was performed using 1 µl of the amplified DNA and a 24 µl master mix (see above).

Primer sequences (Fenner et al. 1991)
CMV-MIEA 6: 5′-AGTGTGGATGACCTACGGGCCATCG-3′
CMV-MIEA 7: 5′-GGTGACACCAGAGAATCAGAGGAGC-3′
Amplification (25 cycles): 94° C – 1 min, 64° C – 1 min, 72° C – 1.5 min.

For optimization of the nested approach the following PCR parameters were tested in serial experiments (Skowasch et al. 1992, Brinkmann and Wiegand 1994):

- Mg^{2+} concentration (1,5; 2,5; 3,5 m*M*),
- primer concentration (0,1; 0,2; 0,4 µ*M*),
- enzyme concentration (0,3; 0,5 U),
- annealing temperature (62, 64, 66° C),
- number of cycles in the nested step (23, 25, 27).

In a series of 50 cases including the cases positive in the lung (detection of the 110 bp nested-fragment) hot start was carried out by heating the samples (containing DNA, 1 primer, aqua bidest and oil overlay) up to 94° C for approximately 3 min, and then pipetting a mixture of the other reaction components through the oil (modified after Chou et al. 1992). The PCR products were separated electrophoretically and visualized using silver staining as described for the PCR approach (Bajanowski et al. 1994). Together with the samples, positive controls (CMV-DNA obtained from a case with histologically defined severe CMV inclusion disease in the salivary glands) as well as blanks (assay lacking DNA extraction) were investigated. To verify the specificity of the 110 bp fragment sequencing of the positive control and 2 other positive samples was carried out (ABI sequencer 373a, ABI, Foster City, USA) using cycle sequencing according to Möller et al. (1994).

Results

Lung: Microscopically no typical changes (inclusion bodies) could be observed. Positive reactions were found for 1 case using ICH and 2 cases by ISH. In 3 cases CMV-DNA could be detected by "conventional" PCR with and without hot start and 7 cases gave positive results using nested PCR (Table 1). Of these 7 cases 3 showed mild forms of interstitial pneumonia.

Submandibular glands: In 10 cases inclusion bodies typical for CMV could be found microscopically. In 13 out of 77 cases positive reactions were found using both IHC and ISH. PCR was successful in 10 and the nested approach in 15 cases.

Liver and kidneys: The specimens investigated each showed 1 positive reaction in the liver and another one in the kidney. Both cases were also positive in the submandibular glands.

Cases positive for IHC (Fig. 1 a) and ISH (Fig. 1 b) in the lungs showed weak reaction patterns compared to the

Table 1 Results of CMV detection in the lung and the submandibular gland using different methods (histology – immunohistochemistry IHC, in situ hybrydization – ISH, PCR, nested PCR) in overview. In brackets the "success rate" of the method compared to nested PCR

Method	CMV positive cases			
	Lung		Submandibular gland	
	N	(Success rate)	N	(Success rate)
Nested PCR	7	(100%)	15	(100%)
PCR*	3	(43%)	10	(67%)
ISH	2	(29%)	13	(**)
IHC	1	(14%)	13	(**)
Histology	0		10	(67%)

* PCR was carried out alternatively with and c/o hot start. The frequency of CMV detection was not influenced by hot start
** IHC and ISH in the submandibular gland were carried out only in 77 cases

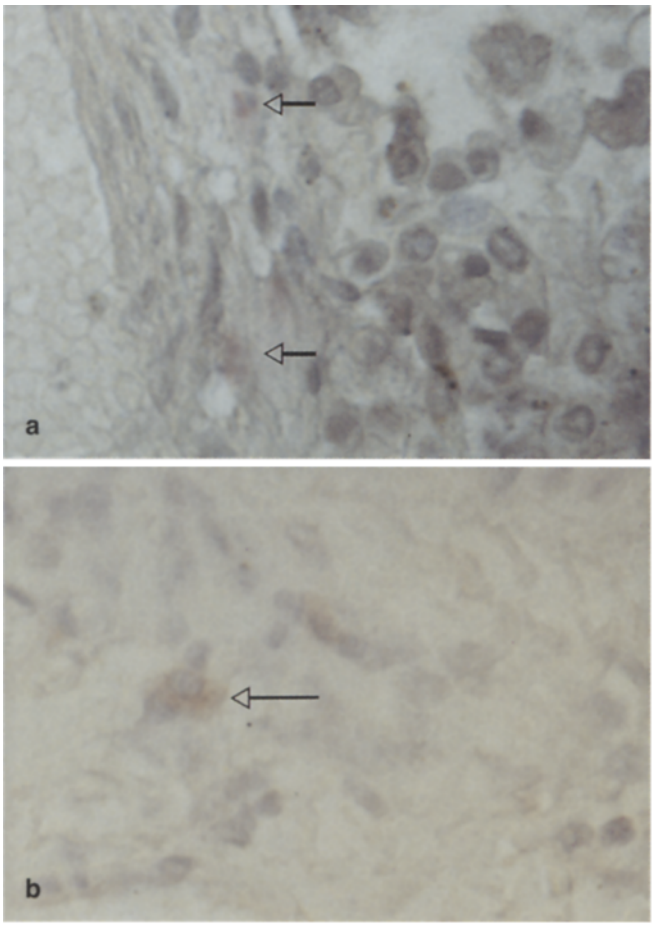

Fig. 1 lung (**a**) Immunohistochemistry: The lung tissue gave positive results *(arrows)* in only 1 case. The staining reaction is weak. (**b**) In situ hybridization: Two cases were positive by ISH, but only a few cells with very weak reactions of the cytoplasm were visible. Morphologically, the positive cells showed no visible changes

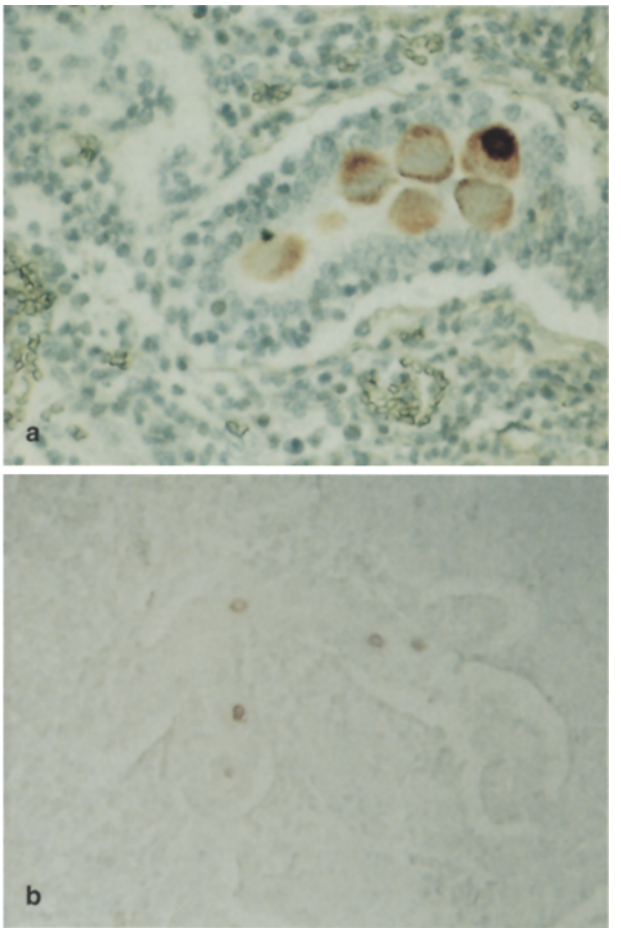

Fig. 2 submandibular gland (**a**) Immunohistochemical detection of CMV antigens in ductal cells showing typical morphological alterations. (**b**) Positive results using ISH

salivary glands (Fig. 2a and 2b). After optimization, nested PCR showed always more distinct bands/patterns with less additional bands compared to conventional PCR (Fig. 3). Hot start resulted in a higher number of additional bands than "pure" PCR and the detection rate could not be increased (Fig. 4). All cases showing positive results for CMV in the lungs were also positive in the submandibular glands. The sequencing results obtained were in accordance to the published DNA sequence of the MIE containing the 110 bp fragment (Stenberg et al. 1984).

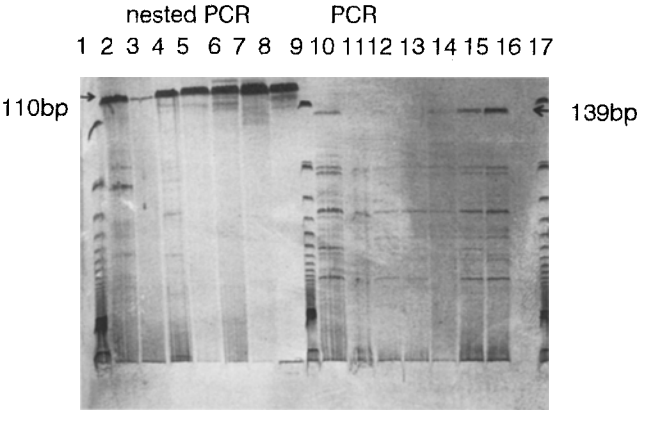

Fig. 3 The positive results obtained from the lung tissue with nested PCR (lanes 2–8) are compared to those with PCR (lanes 10–16). The sequence of the samples on both sides is identical. PCR showed 2 doubtful positive reactions (lanes 12 and 14) and 2 were negative (lanes 11 and 13). Additional bands are less frequent in the nested approach. Lanes 1, 9, 17: 123 bp ladder (GIBCO-BRL, UK) as fragment size control

Discussion

Under experimental conditions the detection of virus DNA in the presence of purified human DNA does not seem to be impaired (Chou et al. 1992). The tissues examined in the present study were autopsy samples collected on average 29 h postmortem with subsequent formalin fixation. DNA degradation due to postmortem influences and depurination in formalin (Sellner 1989) had therefore occured. In the submandibular glands 15 cases

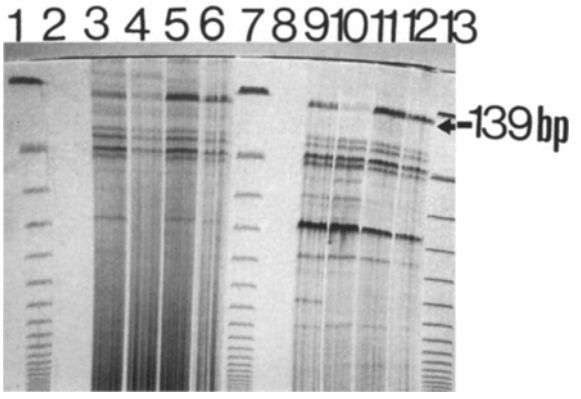

Fig. 4 The results of conventional PCR (lanes 8–12) are compared to PCR with hot start (lanes 2–6). Lanes 2 and 8: negative controls; lanes 6 and 12: positive controls. Hot start showed more additional bands especially in the region near of the 139 bp fragment. The conventional method showed more intensive additional bands with larger fragment length. Lanes 1, 7 and 13: 123 bp ladder as fragment size control

were positive for CMV after applying a variety of methods, but only 10 were positive using conventional PCR although the PCR parameters had been optimized. To further improve these results 3 approaches were tested: 1. an increased number of cycles; 2. hot start (Erlich et al. 1991, Nuovo et al. 1991, Chou et al. 1992); 3. nested PCR (Frank et al. 1992, Collins et al. 1993, Haff 1994). An increase in the number of cycles was associated with an increase in non-specific bands. Although hot start usually leads to a higher sensitivity especially in cases with low copy numbers (Chou et al. 1992), in combination with a reduction of mispriming it did not improve the detection of CMV-DNA in the present study by conventional PCR. In contrast to the nested step, hot start did not influence the disadvantageous relationship between target DNA and background DNA (human and bacterial DNA). In accordance with Frank et al. (1992) and Collins et al. (1993) the best results were obtained using nested PCR because this 2-step procedure can lead to an improvement of the ratio between CMV target DNA and background DNA. The nested PCR approach was more sensitive than conventional PCR (7 positive cases versus 3 in the lung tissue) and more specific resulting in a further reduction of non-specific bands. To demonstrate the specificity of the amplified 110 bp fragment, sequencing was carried out in 2 positive cases as well as in the "CMV control DNA" and identical sequences were found which corresponded to the CMV sequence of the MIE reported by Stenberg et al. (1984). Therefore it can be concluded that the nested approach led to specific CMV-DNA fragments in all positive cases. The advantage of the method applied lies in the detection of CMV genomic DNA in tissues but this is not necessarily clinically relevant. During the viraemia stage, which can last from a few days to several weeks, the virus has been detected in lymphocytes, monocytes and granulocytes (Boland et al. 1992; Revello et al. 1989). At this stage a dissemination of the virus into several organs can occur leading to inflammatory changes (Bruggeman et al. 1985; Griffiths and Grundy 1987). The 3 cases showing an association of CMV detection in the lungs and mild forms of interstitial pneumonia could correspond to a generalized infection but could also be a coincidence. The hypothesis of generalization is more likely in the two cases exhibiting positive results also in the kidney or in the liver. The frequency of CMV detection in the submandibular glands was 12,7% using nested PCR. This level is higher than reported by other authors in a similar group and region (Molz et al. 1985 – 10,4%; Püschel et al. 1988 – 9,1%; Cremer and Althoff 1991 – 4,9%), but these authors investigated the parotis gland by histology, IHC and ISH. In 5–10% of all cases the CMV inclusion disease should be a generalized infection affecting different organs (Stagno et al. 1982, Onorato et al. 1985). Therefore the detection of CMV-DNA in the lung tissue in 7 cases using nested PCR is unexpectedly high showing that the difference in the detection rate comparing PCR and nested PCR is relevant.

In conclusion nested PCR can be used as a tool for the detection of minimal amounts of CMV-DNA. The method leads to a higher detection rate compared to conventional PCR and seems to be especially suitable for formalin fixed autopsy samples with degraded and depurinated DNA.

References

1. Bajanowski T, Wiegand P, Brinkmann B (1994) Comparison of different methods for CMV detection. Int J Legal Med 106: 219–222
2. Boland GJ, De Weger RA, Tilanus MGJ, Ververs C, Bosboom-Kalsbeek K, De Gast GC (1992) Detection of cytomegalovirus (CMV) in granulocytes by polymerase chain reaction compared with the CMV antigen test. J Clin Microbiol 30: 1763–1767
3. Brinkmann B, Wiegand P (1994) Medicolegal implications of PCR based VNTRs. In: Fourth International Symposium on Human Identification 1993. Promega, Madison, pp 149–160
4. Bruggeman CA, Meijer H, Bosman F, Van Boven CPA (1985) Biology of rat cytomegalovirus infection. Intervirology 24: 1–9
5. Cassol SA, Poon MC, Pal R, Naylor MC, Culver-James J, Bowen TJ, Russell JA, Krawetz ST, Pon RT, Hoar DI (1989) Primer-mediated enzymatic amplification of cytomegalovirus (CMV) DNA: application to the early diagnosis of CMV infection in marrow transplant recipients. J Clin Invest 83: 1109–1115
6. Chou Q, Russell M, Birch DE, Raymond J, Bloch W (1992) Prevention of pre-PCR mis-priming and primer dimerization improves low-copy-number amplifications. Nucleic Acid Res 20: 1717–1723
7. Collins T, Pomeroy C, Jordan MC (1993) Detection of latent cytomegalovirus DNA in diverse organs of mice. JID 168: 725–729
8. Costa J, Rabson AS (1985) Viral diseases. In: Kissane JM (ed) Anderson's Pathology. 8. ed. CV Mosby Company, St. Louis Toronto Princeton, pp 360–363
9. Cremer U, Althoff H (1991) Nachweis und Inzidenz von Zytomegalievirusinfekten bei plötzlichen Kindstodesfällen (SIDS). Rechtsmedizin 1: 25–28
10. Erlich HA, Gelfand D, Sninsky JJ (1991) Recent advances in the polymerase chain reaction. Science 25: 1643–1651
11. Fenner TE, Garweg J, Hufert FT, Boehnke M, Schmitz H (1991) Diagnosis of human cytomegalovirus-induced retinitis in human immunodeficiency virus type1-infected subjects by using the polymerase chain reaction. J Clin Microbiol 29: 2621–2622

12. Frank TS, Cook SM, Del Buono EA, Wilson M (1992) A simplified method for detecting Cytomegalovirus by polymerase chain reaction from histologic sections of small biopsies. Modern Pathology 4:449–454
13. Griffiths PD, Grundy JE (1987) Molecular biology and immunology of cytomegalovirus. Biochem J 241:313–324
14. Haff LA (1994) Improved quantitative PCR using nested primers. PCR Meth Appl 3:332–337
15. Höfler H, DeLellis RA, Wolfe HJ (1988) In situ hybridization and immunohistochemistry. In: DeLellis RA (ed) Advances in immunohistochemistry. Raven Press, New York, pp 47–66
16. Jiwa NM, Steenberg RDM, Zwaan FE, Kluin PM, Raap AK, van der Ploeg M (1989) Three sensitive methods for the detection of cytomegalovirus in lung tissue of patients with interstitial pneumonitis. Am J Clin Pathol 93:117–124
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. Molz G, Hartmann HP, Michels L (1985) Plötzlicher Kindstod: Histologische Befunde in den Kopfspeicheldrüsen. Pathologe 6:8–12
19. Nuovo GJ, Gallery F, MacConnell P, Becker J, Bloch W (1991) An improved technique for the in situ detection of DNA after polymerase chain reaction amplification. Am J Path 139: 1239–1244
20. Onorato JM, Morens DM, Martone WJ, Stansfield SK (1985) Epidemiology of cytomegaloviral infections: recommendations for prevention and control. Rev Infect Dis 7:479–497
21. Püschel K, Hashimoto Y, Löning T, Lignitz E (1988) Cytomegalie der Kopfspeicheldrüsen bei SIDS. Z Rechtsmed 99: 281–289
22. Revello MG, Percivalle E, Zavattoni M, Parea M, Gross P, Gerna G (1989) Detection of human cytomegalovirus immediate-early antigen in leukocytes as a marker of viremia in immunocompromised patients. J Med Virol 29:88–93
23. Sellner W (1989) Fixierung histologischer Präparate. In: Böck P (ed) Mikroskopische Technik/Romeis. 17. ed, Urban and Schwarzenberg, München Wien Baltimore, p 83–87
24. Skowasch K, Brinkmann B (1992) Optimized amplification of the polymorphic system COL2A1. Int J Legal Med 105:185–187
25. Stagno S, Pass RF, Dworsky ME, Henderson RE, Moore EG, Walton PD, Alford CA (1982) Congenital cytomegalovirus infection. The relative importance of primary and recurrent maternal infection. N Engl J Med 306:945–949
26. Stenberg RM, Thomsen DR, Stinski MF (1984) Structural analysis of the major immediate early gene of human cytomegalovirus. J Virol 49:190–194
27. Wolfe HJ, Ross D, Wolfe B (1990) Detection of infectious agents by molecular methods at the cellular level. Verh Dtsch Ges Path 74:295–300
28. Wu GD, Shintaku IP, Chien K, Geller SA (1989) A comparison of routine light microscopy, immunohistochemistry, and in situ hybridization for the detection of cytomegalovirus in gastrointestinal biopsies. Am J Gastroenterol 84:1517–1520