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Comparison of different methods for CMV detection

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Summary. The submandibular glands were investigated in 77 cases of SIDS using histology, immunohistochemistry (IHC), in situ hybridization (ISH) and PCR to detect cytomegalovirus (CMV) inclusion disease. The aim of the investigation was to establish the detection of CMV by PCR as a model for the detection of other types of virus by PCR, especially for the detection of viruses which affect the respiratory system of babies. In 14 cases the CMV detection was possible. Typical changes of duct cells associated with CMV infection were observed in 9 by histology and in 4 further cases by IHC. In comparison to these methods, ISH showed a higher sensitivity but only one more positive case was found. Theoretically the PCR method is more sensitive than ISH and more convenient from the practical aspect. Detection of CMV by PCR was possible in 10 out of 14 positive cases. All PCR positive cases were cases showing an active disease but not latent infection.

Key words: SIDS – Cytomegalovirus inclusion disease – Submandibular gland – In situ hybridization – Polymerase – Chain reaction

Zusammenfassung. In 77 SIDS Fällen wurden die Glandulae submandibularis zum Nachweis einer Zytomegalievirusinfektion (CMV) mit verschiedenen Methoden (Histologie, Immunhistochemie [IHC], In situ Hybridisierung [ISH] und PCR) untersucht. Ziel der Untersuchung war gleichzeitig, den CMV-Nachweis mittels PCR als Modell zum Nachweis anderer Virustypen, insbesondere mit Affinität zum respiratorischen System von Säuglingen, zu etablieren. In 9 von insgesamt 14 CMV-positiven Fällen zeigte die Histologie typische Eulenaugenzellen. In weiteren 4 Fällen ergab die IHC einen Virusnachweis. Ein zusätzlicher Informationsgewinn durch ISH war dagegen nur in einem weiteren Fall möglich. Die PCR ist zweifellos die Methode mit der höchsten Sensitivität. Ein Virusnachweis gelang jedoch nur in 10 der positiven Fälle. In den Fällen mit latenter Infektion konnte trotz Methodenoptimierung ein CMV-Nachweis nicht erreicht werden. Eine mögliche Ursache dafür könnte in einer für den CMV-Nachweis nicht optimalen Primer-Sequenz bestehen.

Schlüsselwörter: SIDS – Zytomegalievirusinfektion – Glandula submandibularis – In situ Hybridisierung – Polymerase – Kettenreaktion

Introduction

Cytomegalovirus (CMV) inclusion disease is an ubiquitious infection and the prevalence of antibodies in adults is in the range 40–100% (Costa and Rabson 1985). Prenatal or postnatal infection produces a disease that may vary from asymptomatic to severe, and occasionally even to fatal course. Also, in 5–10% of all cases CMV inclusion disease is a generalized infection affecting different organs such as brain, liver, kidneys, lungs and eyes (Stagno et al. 1982; Onorato et al. 1985). In recent years an increase of infections of salivary glands by sialotropic viruses has been reported (Molz et al. 1985; Püschel et al. 1988).

In histological sections CMV-positive cases show typical changes of ductal cells of salivary glands (Costa and Rabson 1985). Although these changes are highly indicative of CMV inclusion disease they cannot be considered as proof (Cremer and Althoff 1991). Furthermore, their presence does not necessarily indicate all infection of this type. This study therefore aims at a systematic comparison of specific (immunohistochemistry, in sity hybridization, in vitro PCR) and nonspecific (histology) detection methods. Furthermore, the sensitivity and specificity of the more advanced methods could also stimulate attempts to demonstrate other types of virus antigens.

Material and methods

The submandibular glands were investigated in 77 fatalities due to Sudden Infant Death Syndrome (SIDS). This diagnosis was established only after thorough post mortem examination, including extensive histology and toxicology. The fatalities showed an age distribution typical for SIDS (Fig. 1). Fixation of the tissue specimens was performed in 4% buffered formalin. After 1–4 days the tissue was embedded in paraffin and prior to the following procedures 4 µm sections were prepared.

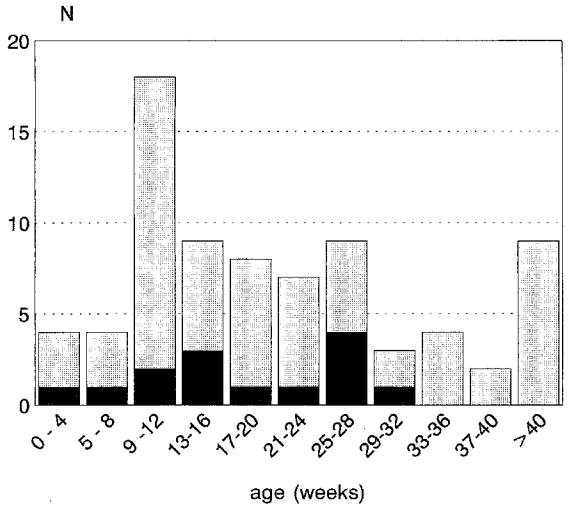

Fig. 1. Age distribution of CMV positive and negative cases. □ negative; ■ positive

1. Histology. Histological investigations were carried out after haematoxylin-eosin staining of the specimens. The cases were divided into 3 groups: (1) without pathological changes; (2) with unspecific infiltration; (3) with changes typical for CMV.

2. Immunohistochemistry. The immunohistochemical detection of the CMV antigens was carried out using a monoclonal antibody (DAKO-CMV, CCH2). The antibody reacts with infected cells giving a nucleus staining pattern with the early antigen and a cytoplasmic reaction with the late antigen (Zweygberg Wirgat et al. 1990; Niedobitek et al. 1988). Prior to the antibody reaction, the tissue sections were preincubated with trypsin for 30 min at 37°C. The antibody was diluted 1:25 and the binding reaction took place at 3°C overnight. Staining was carried out using the Avidin-Biotin technique (Nishi et al. 1988).

3. In situ hybridization (ISH). The DNA probe assay from ENZO-Diagnostics was used for the identification of CMV. The detection of CMV was carried out according to the manufacturers instructions with the following modifications:

(1) deparaffination including treatment with NH_3-alcohol
(2) to inactivate endogenous peroxidase activity 1% H_2O_2-methanol was used for 30 min
(3) digestion of the tissue was carried out with proteinase K (Sigma, 0.01%, pH 7.5) for 30 min at 37°C
(4) hybridization at 37°C in a wet box for 60 min
(5) counter staining with diluted haemalaun.

4. Polymerase chain reaction. PCR was carried out according to Chen et al. (1992) with some modifications. The CMV DNA sequence chosen for amplification is derived from the late antigen GP 64 region and has a length of 139 bp (Shibata et al. 1988; Chen et al. 1992). Tissue sections were deparaffinized by xylol/ethanol. The DNA digestion was performed using proteinase K at 37°C overnight. Proteinase K was inhibited by boiling the sample extract for 10 minutes.

PCR protocol: 1 µl from 50 µl extraction volume in dilutions of 1:10 and 1:100 was used as template DNA. 1 U Taq polymerase (Promega), 0.3 µ*M* each primer, 50 µ*M* of each dNTP, 2 µl PCR buffer (Promega) diluted to a total volume of 25 µl with distilled water. The reaction mixture was overlayed with 2 drops of oil.

Primer sequence (Chen et al. 1992)
5′-CCG CAA CCT GGT GCC CAT GG-3′
5′-CGT TTG GGT TGC GCA GCG GG-3′

Amplification conditions:
94°C – 1 minute (denaturation),
60°C – 1 minute (annealing),
70°C – 1 minute (extension);
30 cycles (Thermocycler Perkin-Elmer Cetus 9600).

Electrophoresis was carried out using polyacrylamide gels with a separation distance of 10 cm. Gel: acrylamide (8% T, 3% C, 400 µm thick) with piperazine diacrylamide as cross-linker using a discontinuous buffer system (Allen et al. 1989); 2 agarose plugs with 0.14 *M* Tris borate and bromophenol blue; 33 m*M* Tris sulfate (pH 9.0) as leading ion.

Electrophoretic parameters: 1000 V, 20 mA, 10 W; electrophoresis was stopped when the bromophenol blue front reached the anodal plug. The CMV fragment was visualized by silver staining (Budowle et al. 1991).

The 123 bp ladder from Gibco BRL was used as control.

Results

Histology

Microscopically the inclusions in the salivary glands are round, highly refractile, homogenous and eosinophilic bodies either in the cytoplasm or in the nucleus of duct cells. An infiltration of mononuclear inflammatory cells was also observed. In 51 cases no pathological changes were observed. In 17 cases unspecific infiltration by mononuclear inflammatory cells and in 9 cases changes typical for CMV could be demonstrated (Fig. 2).

Immunohistochemistry

Positive staining could be observed in 13 cases (Fig. 3). Not all cells containing inclusion bodies showed a positive reaction. In 4 cases there were some cells with no visible inclusion bodies but positive staining reactions.

In situ hybridization

Using ISH the detection of viral DNA was possible in 13 cases (Fig. 4) including 1 case which gave negative stain-

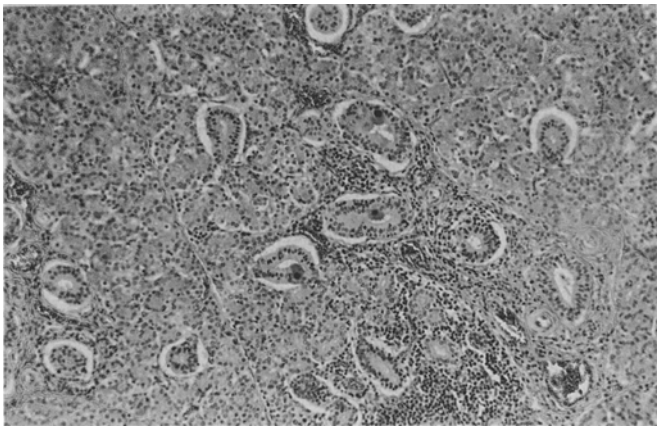

Fig. 2. Typical CMV affected ductal cells of the submandibular gland with increased size and inclusion bodies. (HE, 40 ×)

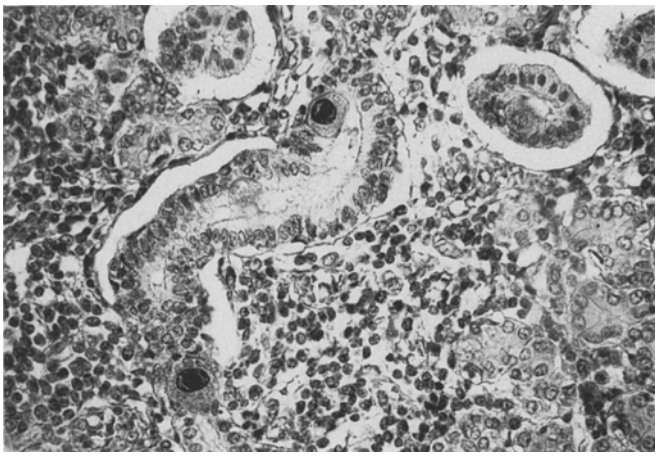

Fig. 3. Immunohistochemistry using avidin – biotin complex method. Positive staining reaction of nuclear inclusion bodies in ductal cells. (100 ×)

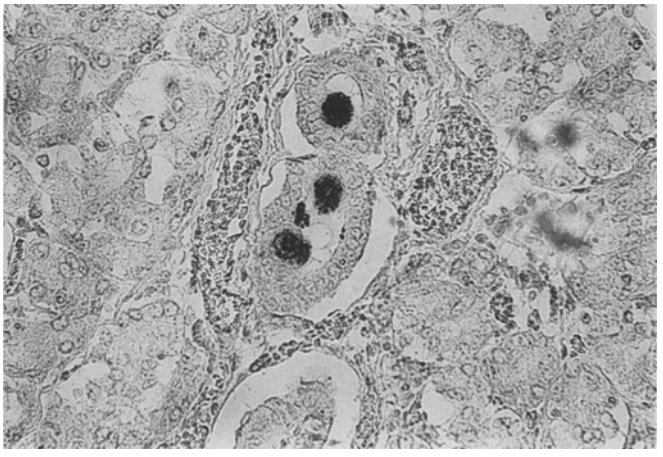

Fig. 4. In situ hybridization. Detection of CMV DNA in the cytoplasm and nucleus of duct cells. (100 ×)

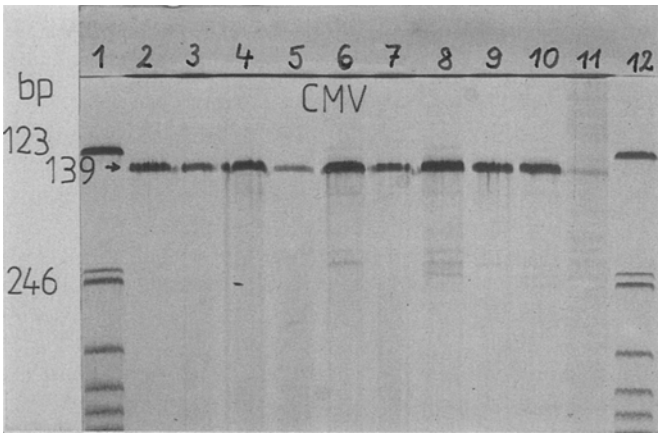

Fig. 5. Detection of CMV by PCR followed by polyacrylamide gel electrophoresis and silver staining. The 123 bp ladder from Gibco BRL is shown in lane 1 and 12. The 139 bp fragment in the other lanes indicates the presence of viral DNA. In lanes 6–11 there are also additional bands

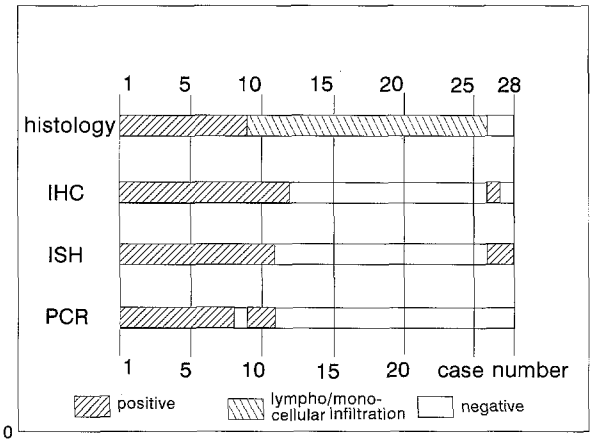

Fig. 6. Comparison of CMV detection by different methods. The 28 cases with conspicious histological findings or with positive results in CMV detection by IHC, ISH or PCR are given in overview. The remaining cases (n = 49) which showed negative results by all methods are not shown

ing in immunohistochemistry. In 1 other case positive IHC reaction could not be confirmed using ISH.

PCR

In 8 out of 9 cases with positive staining in IHC, ISH and with typical histological findings the PCR was positive showing the expected 139 bp amplified product by silver staining (Fig. 5). 2 other cases with positive reactions only by IHC and ISH also showed positive results in PCR. In all other cases the detection of CMV DNA by PCR was not possible. Changes of the conditions for DNA extraction and PCR showed no improvement in the results.

The number of CMV positive cases in the age groups up to 32 weeks showed only small differences. The highest incidence of CMV inclusion disease was found in the age group 25–28 weeks (45%).

In summary 14 out of the 77 cases showed CMV inclusion disease or a detection of cytomegaloviruses (Fig. 6) giving an incidence of ca. 18% among the children investigated.

Discussion

The incidence of CMV among the children investigated in this study is higher than reported by other authors (Molz et al. 1985; Püschel et al. 1988; Cremer and Althoff 1991), but in these studies the parotid gland was investigated.

A comparision of the 4 different methods showed that histology and IHC are sufficient for the detection of CMV in routine work. Detection was possible by these 2 methods in 13 out of 14 positive cases. The additional information obtained by ISH is relatively small. In the ISH kit the length of DNA probes varied between 2.3 and 13.2 kb. Therefore the ISH also depends on the degree of degradation of DNA, which in the postmortem period can either be affected by enzymatic activation or by prolonged fixation in formalin.

Thus the main advantage of ISH lies in it's specificity in combination with the localization of the viruses. The levels of sensitivity of ISH and IHC seem to be similar. Although Keh and Gerber (1988), Wu et al. (1989) and Cremer and Althoff (1991) pointed out it's usefulness, ISH seems to offer only limited additional advantages and can therefore be useful under special questions (Jiwa et al. 1989; Clayton et al. 1989).

PCR is without any doubt the most sensitive method. Theoretically it should be possible to detect 1 virus in 40000 cells (Casoll et al. 1989). In practice CMV detection by PCR was successful in only 70% of our cases which showed positive results using other techniques. This result corresponds to the experience of Chen et al. who were able to detect CMV only in cases of active disease but not latent infection. In order to optimize the PCR assay we mainly changed the annealing temperature, the number of cycles and the amount of template DNA. By increasing the number of cycles it was not possible to reach a higher sensitivity. Especially in cases where latent infection was assumed only additional unspecific fragments could be observed. The annealing temperature of 60° C seems to be optimal. A higher temperature led to a reduction in sensitivity. Effects of degradation could be excluded because of positive reactions using ISH which detects only much longer fragments. The possible influence of an incomplete digestion was excluded by a prolonged digestion time up to 3 days and inhibition of the PCR by remnants of paraffin could be excluded by using phenol/chloroform/isoamylalcohol (24:24:1) for DNA extraction. Furthermore, it was possible to show that substrate excess led to an inhibition of the PCR. Chen et al. (1992) recommended therefore titration of the substrate using positive samples. Other causes of reduced PCR sensitivity have to be elaborated, e.g. suitability of the primer sequences chosen, inhibition by background DNA etc.

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