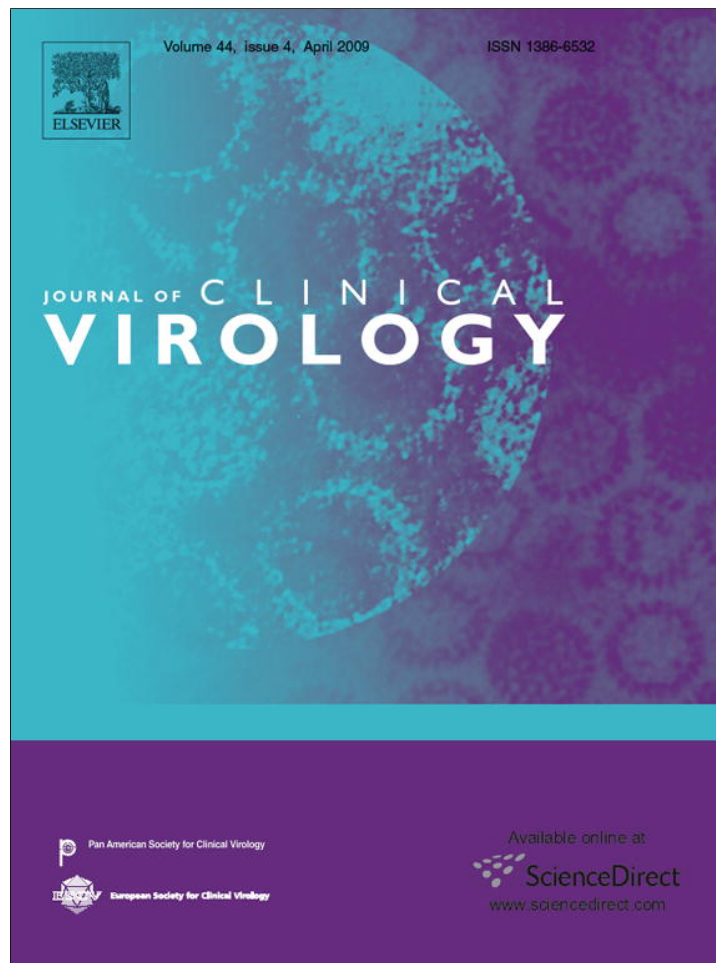


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Detection of herpes viruses in children with acute appendicitis

Polixeni Katzoli^a, George Sakellaris^b, Maria Ergazaki^a, George Charissis^b,
Demetrios A. Spandidos^a, George Sourvinos^{a,*}

^a Laboratory of Virology, Faculty of Medicine, University of Crete, Heraklion 71003, Crete, Greece

^b Department of Paediatric Surgery, University Hospital, Heraklion 70014, Crete, Greece

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ABSTRACT

Objective: This study aimed to investigate the incidence of herpes simplex virus (HSV) types-1 and -2, varicella-zoster virus (VZV), cytomegalovirus (CMV), Epstein-Barr virus (EBV), human herpes virus 6 (HHV-6) and human herpes virus 7 (HHV-7) in childhood acute appendicitis.

Study design: Polymerase chain reaction (PCR) assays were applied to detect herpes virus DNA in 38 children [11 girls and 27 boys, mean age 9 years (STD ± 2.59), range 6–14 years], who underwent an appendectomy within a 2.5-year period. Appendix, omentum and peripheral blood mononuclear cells (PBMCs) were available from each case. Of the 38 children with acute appendicitis, 20 (52.6%) had advanced (phlegmonous) acute appendicitis and 18 (47.4%) had perforated appendicitis and local peritonitis. Forty-one blood specimens from age-matched healthy children (25 female and 16 male), with clinical manifestations unrelated to viral infections served as negative controls.

Results: CMV was the most frequently detected virus (8/38, 21%), followed by HHV-6 (3/38, 7.9%). EBV and HSV-1 were detected, though not in all three different types of tissue specimens tested. None of the samples examined were HSV-2-, VZV- or HHV-7-positive. Of all the specimens, the omentum was the most commonly infected tissue (63.0%) while the appendix and peripheral blood specimens were found to be positive for viral infection in 60.5% and 50% of cases, respectively. The CMV IgG+ antibodies were positive in 54% of the control cases while 86% of the same group presented HHV-6 IgG+ antibodies.

Conclusion: To the best of our knowledge, this is the first study documenting the presence of herpes virus DNA in children with acute appendicitis, suggesting that possible viral infection or reactivation is associated with childhood appendicitis.

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1. Introduction

Acute appendicitis, an inflammation of the appendix, is one of the most common reasons for emergency abdominal surgery in children.¹ Appendicitis primarily affects adolescents and young adults, with a peak incidence during the second and third decades of life. Among children operated for the disease, 77% are between 6 and 18 years of age, and only 2% are younger than 3 years of age.^{1–3} Among teenagers and young adults, males are more commonly affected than females, with a male to female ratio of approximately 1.3:1. However, the gender distribution is uniform beyond these age groups.^{1,4,5}

Abbreviations: HSV-1, Herpes Simplex Virus type-1; HSV-2, Herpes Simplex Virus type-2; CMV, Cytomegalovirus; VZV, Varicella Zoster Virus; EBV, Epstein Barr Virus; HHV-6, Human Herpes Virus type-6; HHV-7, Human Herpes Virus type-7; PCR, Polymerase Chain Reaction.

* Corresponding author. Tel.: +30 2810 394835; fax: +30 2810 394759.

E-mail address: sourvino@med.uoc.gr (G. Sourvinos).

Appendicitis results from the luminal obstruction of the appendiceal lymphoid follicle by fecaliths hyperplasia, carcinoid tumours or foreign bodies.^{1,6} Additionally, bacterial infections such as *Bacteroides fragilis*, *Escherichia coli*, *Yersinia*, *Salmonella* or *Shigella*, parasitic infestations such as *Entamoeba histolytica*, or enteric and systemic viral diseases such as measles virus, adenovirus and cytomegalovirus (CMV) infection, can cause a reaction of the lymphoid follicle.^{7–21}

White blood cell count and C-reactive protein concentrations in serum play only a supportive role in the diagnosis of acute appendicitis in children.^{22,23} Among other laboratory methods for the detection of acute appendicitis, new inflammatory markers such as interleukin (IL)-6, lipopolysaccharide-binding protein (LBP) and nuclear factor (NF)-kappaB have been studied. However, their diagnostic value remains controversial.^{23–27}

Herpes viruses are a diverse family of large DNA viruses, all of which have the capacity to establish lifelong latent infections.²⁸ Primary infection with many of these viruses is common during childhood. However, their role in acute appendicitis is poorly understood. The disease has been reported in the setting of CMV

reactivation or acute CMV infection in adult patients with AIDS.²⁹ Furthermore, acute appendicitis was observed to follow acute EBV infection in an immunocompetent patient.¹⁶ An earlier report implicated CMV in acute appendicitis in a non-immunosuppressed non-HIV homosexual patient.³⁰ In contrast, Adcock et al. described a case of pseudoappendicitis preceding infectious mononucleosis where the patient presented typical symptoms of acute appendicitis but was found, at laparoscopy, to have mesenteric adenitis.³¹

This study aimed to investigate the potential role of seven herpes viruses, HSV-1, HSV-2, VZV, CMV, EBV, HHV-6 and HHV-7, in the pathogenesis of acute appendiceal diseases since (a) primary infection with these viruses occurs mostly in childhood and (b) certain other viruses, especially those of the upper respiratory system, are known to precede appendicitis.^{14,13,32} A molecular analysis of the appendix, omentum, and peripheral blood samples revealed a considerable frequency of CMV and HHV-6 DNA in childhood acute appendicitis, implying that viral infection plays an important role in the pathogenesis of the disease.

2. Methods

2.1. Patients

The present study included 38 cases of acute appendicitis in children with a mean age of 9 years ($STD \pm 2.59$ years) and a range of 6–14 years. Children underwent an appendectomy within a 2.5-year period, and samples were obtained from the Surgical Paediatric Clinic, University Hospital of Heraklion, Crete, Greece. The ethics committee of the university approved the study, and the participating donors gave their written informed consent. The study conformed to the principles outlined in the Declaration of Helsinki. Female children comprised 29.0% (11/38) of the patients, while 71% (27/38) were male. Of the 38 children with acute appendicitis, 20 (52.6%) had advanced (phlegmonous) acute appendicitis without perforation, and 18 (47.4%) had perforated appendicitis and local peritonitis. The initial paediatric surgical evaluation consisted of history, physical examination, white blood cell count, differential count and urinalysis. Imaging was used selectively by the paediatric surgeon. Children diagnosed with appendicitis without perforation underwent an appendectomy without additional testing. Children with perforated appendicitis received more intensive preparation, with intravenous hydration and antibiotics prior to the operation. The final diagnosis was based on histology and, in the case of perforation, on a macroscopic evaluation by the surgeon.

2.2. Specimens

Three types of samples were obtained from each case: (a) a section of vermiform appendix, (b) a section of the omentum and (c) a sample of peripheral blood collected in EDTA anticoagulant tubes. Tissue samples were placed in sterilized polypropylene 1.5 ml tubes. Appendix, omentum and peripheral blood samples from 38 cases [20 of advanced (phlegmonous) acute appendicitis and 18 of perforated appendicitis and local peritonitis] were obtained. In addition, 41 blood specimens from 9-year-old healthy children (25 female and 16 male) ($STD \pm 2.98$ years) with clinical manifestations unrelated to viral infections served as negative controls. Tissue samples were stored at -80°C , and the EDTA blood samples at 4°C until DNA extraction and PCR. The detection of HSV-1, HSV-2, CMV, EBV, VZV and HHV-6 IgG antibodies from the plasma of the healthy children enrolled in the study was carried out using a standard enzyme immunoassay.

2.3. DNA extraction

DNA was extracted from the tissue samples and peripheral blood by proteinase K treatment and phenol–chloroform extraction. After

ethanol precipitation, DNA was suspended in H_2O and stored at -20°C until use. DNA sample concentrations were determined using a spectrophotometer (260 nm) prior to PCR amplification.

2.4. Polymerase chain reaction (PCR)

Single and nested PCR assays were performed to detect HSV-1, HSV-2, VZV, CMV, EBV, HHV-6 and HHV-7 as previously described.³³ Particular care was taken and all manipulations were performed inside a PCR-hood to avoid potential contamination. The PCR products were examined by electrophoresis in a 2% or 3% agarose gel, depending on their size, and photographed on an ultraviolet light transilluminator. The integrity and quality of the extracted DNA were confirmed after the successful amplification of the *beta2-microglobulin* gene in all of the samples. The sensitivity of our PCR assay was determined by applying a serial-dilution amplification assay of viral positive control DNAs with a known number of copies.³⁴

To distinguish between HSV-1 and -2, and confirm the specificity of the PCR product for all of the viruses tested, the PCR products were digested with appropriate restriction enzymes. Finally, these viral PCR products were subjected to direct sequencing analysis to verify the initial amplification results.

2.5. Statistical analysis

The association between the viral status of the samples and the clinical stage of acute appendicitis, as well as the association with tissue type, was examined using Student's *t*-test (after examination for equality of variance with Levene's test) or its non-parametric equivalents, the Mann–Whitney *U* and Kruskal–Wallis *H* tests. Statistical analyses were 2-sided and performed with SPSS 11.5 (SPSS, Chicago, IL). Statistical significance was set at the 95% level ($p < 0.05$).

3. Results

Thirty-eight patients at different clinical stages of the disease, including 20 cases with advanced (phlegmonous) acute appendicitis and 18 with perforated appendicitis and local peritonitis, were screened for the presence of HSV-1, HSV-2, VZV, CMV, EBV, HHV-6 and HHV-7 DNA. A total of 114 human specimens obtained from different tissues, including 38 samples from the appendix, 38 from the omentum and 38 from the peripheral blood, were tested by single or nested PCR-based assays. The cumulative results of the PCR analysis are shown in Table 1 and representative examples of PCR-positive samples are shown in Fig. 1. The frequency of viral DNA detection in the three different sites is presented in Table 2.

Among the 38 children, 28 (73.7%) were positive for the presence of CMV DNA in at least one of the tissue specimens tested (Table 3). Specifically, 3/28 cases presented CMV DNA in the appendix alone (A), while 7/28 and 6/28 were positive in just the omentum (O) and just the peripheral blood (B), respectively. In 2/28 children, CMV DNA was detected in both the appendix and the omentum (A+O), while 2 additional cases were CMV-positive in both the omentum and the peripheral blood (O+B). Finally and most importantly, CMV DNA was detected in all 3 types of specimens (A+O+B) in 8/28 children. In terms of the clinical stage of the disease, of the 28 CMV-positive cases, 17 (61.0%) presented advanced (phlegmonous) acute appendicitis and 11 (39.2%) exhibited perforated appendicitis and local peritonitis. HCMV IgG+ antibodies were detected in 54% of the control cases.

A considerably high incidence was also found for HHV-6. HHV-6 DNA was detected in at least one of the tissue samples tested in 18 cases (47.4%) (Table 3). Ten of 18 children were positive in the appendix (A) exclusively and 4/18 in the omentum alone, while only

Table 1
Cumulative results of viral detection in children with acute appendicitis and perforated appendicitis and local peritonitis.

Virus		Acute appendicitis			Perforated appendicitis-local peritonitis		
		No. of patients	Viral frequency in patients (N = 20)	Frequency in PCR positive and negative patients	No. of patients	Viral frequency in patients (N = 18)	Frequency in PCR positive and negative patients
CMV	Positive	17	85%	61% (N = 28)	11	61%	39% (N = 28)
	Negative	3	15%	30% (N = 10)	7	39%	70% (N = 10)
HHV-6	Positive	10	50%	36% (N = 18)	8	44%	44% (N = 18)
	Negative	10	50%	50% (N = 20)	10	56%	50% (N = 20)
EBV	Positive	2	10%	67% (N = 3)	1	6.0%	33% (N = 3)
	Negative	18	90%	51% (N = 35)	17	94%	49% (N = 35)
HSV-1	Positive	1	5%	50% (N = 2)	1	6.0%	50% (N = 2)
	Negative	19	95%	53% (N = 36)	17	94%	47% (N = 36)
HSV-2	Positive	0	0%	0% (N = 0)	0	0%	0% (N = 0)
	Negative	20	100%	53% (N = 38)	18	100%	47% (N = 38)
VZV	Positive	0	0%	0% (N = 0)	0	0%	0% (N = 0)
	Negative	20	100%	53% (N = 38)	18	100%	47% (N = 38)
HHV-7	Positive	0	0%	0% (N = 0)	0	0%	0% (N = 0)
	Negative	20	100%	53% (N = 38)	18	100%	47% (N = 38)

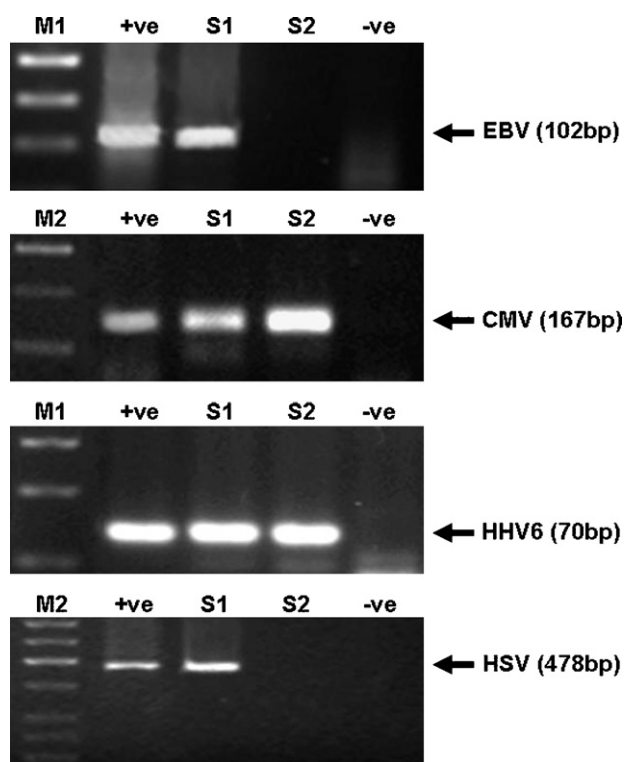


Fig. 1. PCR detection of herpes viruses HSV-1, EBV, CMV and HHV-6 in acute appendicitis samples. PCR products were visualized in 2% agarose gel. Lanes S1–S2: appendicitis samples; +ve: positive control; –ve: negative control and M: molecular weight marker.

Table 2
Frequency of viral DNA detection in the three different sites.

Tissue type		No. of samples	Frequency
Appendiceal	Positive	23	60.5%
	Negative	15	39.5%
	Total	38	100%
Omentum	Positive	24	63%
	Negative	14	37%
	Total	38	100%
Peripheral blood	Positive	19	50%
	Negative	19	50%
	Total	38	100%

one patient tested positive in both the appendix and the omentum. It should be emphasized that three patients were HHV-6 DNA-positive in all three tissue samples examined (A + O + B). Ten the 18 patients (55.5%) presented advanced (phlegmonous) acute appendicitis and 8 (44%) presented both perforated appendicitis and local peritonitis. The immunoassays revealed a significantly high proportion of HHV-6 IgG+ antibodies (86%) in the control group.

Interestingly, the PCR analysis revealed that one of the children was positive for both CMV and HHV-6 virus.

The prevalence of EBV and HSV-1 was very low, and the detection of viral DNA was restricted to single types of tissue specimens (Table 3). In particular, 3 children were EBV-positive (7.9%) 2 of them in the omentum alone and 1 in the peripheral blood alone, while HSV-1 DNA was exclusively detected in the appendix of only 2 patients (5.3%) EBV IgG+ or HSV-1 IgG+ subjects were detected in 48% and 37% of the control cases, respectively.

PCR analysis for HSV-2, VZV and HHV-7 proved negative for all the samples tested.

Overall, viral DNA was detected most frequently in the omentum (63.0%) followed by the appendicitis (60.5%) and the peripheral blood (50.0%).

Table 3
Frequency of viral DNA detection in the tissue samples of children with acute appendicitis.

Virus	No. of patients tested	No. of positive patients	No. of negative patients	A	O	B	A + O + B	A + O	A + B	O + B
CMV (%)	38	28 (73.7)	10 (26)	3/28 (10.7)	7/28 (25)	6/28 (21.4)	8/28 (28.6)	2/28 (7.1)	–	2/28 (7.1)
HHV-6 (%)	38	18 (47.4)	20 (53)	10/18 (55.5)	4/18 (22)	–	3/18 (16)	1/18 (5.5)	–	–
EBV (%)	38	3 (7.9)	35 (92)	–	2/3 (67)	1/3 (33)	–	–	–	–
HSV-1 (%)	38	2 (5.3)	36 (95)	2/2 (100)	–	–	–	–	–	–

A: appendicitis; O: omentum; B: blood.

All CMV-positive children presented similar clinical features, compatible with CMV infection. Specifically, of the seven patients who were only positive for the presence of CMV DNA, four experienced fever and generalized adenopathy, two had fever, liver and spleen enlargement, and the last had atypical lymphocytes. One of the two children who tested positive for the presence of HHV-6 DNA alone presented a generalized rash, while the other had generalized adenopathy and fever. A last child who was positive for both CMV and HHV-6 DNA presented generalized adenopathy along with retinitis. No statistically significant association was observed between the presence of viral DNA and the clinical stage of the acute appendicitis or the different tissue types.

4. Discussion

Although acute appendicitis is one of the most common surgical diseases in children, information regarding the implications of pathogens in the disease remains extremely limited. The existing evidence mainly concerns bacterial infections such as *B. fragilis*, *E. coli*, *Yersinia*, *Salmonella* and *Shigella*, and parasitic infestations such as *E. histolytica*.^{7–13} In terms of viruses, the literature is generally restricted to case studies, implicating viruses such as measles, adenovirus or cytomegalovirus in immunocompromised patients.^{14–21}

Herpes viruses are ubiquitous pathogens in children, remaining quiescent after an active infection. Their high prevalence in childhood prompted us to investigate their possible role in the pathogenesis of acute appendicitis, based on the hypothesis that the herpes viruses are latent in the appendix, and are periodically reactivated by certain triggers such as individual immune status, stress or sunlight. This may cause an acute viral infection at the time of or just before the onset of appendicitis, which leading to lymphoid hyperplasia in the appendix. This hyperplasia or subsequent healing and scarring may produce an acute obstruction of the appendix. In the case of infection by herpes viruses, we would expect some evidence of viral DNA in either the appendix or omentum, or even in the peripheral blood.

The PCR analysis revealed that CMV exhibited the highest incidence (21%) among the herpes viruses in children with acute appendicitis. CMV appendicitis has been reported in the setting of CMV reactivation or acute CMV infection in patients with acquired immune deficiency syndrome (AIDS).²⁹ An earlier report implicated CMV in acute appendicitis in a non-immunosuppressed non-HIV homosexual patient.³⁰

HHV-6, one of the most recently discovered herpes viruses, is also highly prevalent (7.8%) in childhood acute appendicitis. Approximately 50–60% of children are infected by HHV-6 by 12 months of age and almost all children are infected by the age of 2–3 years. Most HHV-6 infections in children are believed to be due to the HHV-6 variant B (HHV-6B), which has been isolated from the peripheral blood leukocytes (PBLs) of healthy children with primary infection.^{35,36} HHV-6 is suspected to be the etiopathological cause of other childhood acute diseases, such as acute lymphoblastic leukemia³⁷ or acute liver failure.³⁸ This implicates the virus in an additional childhood acute disease.

The percentages of CMV and HHV-6 IgG+ antibodies were found to be significantly high in the control group. Given these high serology results, the most likely explanation for the high incidence of CMV and HHV-6 detected by PCR is that the children have experienced a reactivation of CMV or HHV-6 which is associated with appendicitis. Quantitative viral load results may provide further support for pathogenesis since it is possible that the inflammatory cells at the site of appendicitis harbored significant copies for herpes virus family DNA.

The epithelial cells are considered to be the principal site of pathogen infection, playing a central role in the viral modulation of

inflammation via the release of a variety of cytokines, chemokines and growth factors. The detection of viral DNA in the appendix suggests that this tissue serves as a host for herpes viruses, causing epithelial alterations. Therefore, the mechanisms by which viral infections modulate epithelial function are the subject of intense investigation. The omentum is a specific fold of peritoneum passing between the stomach and other abdominal viscera. As with other adipose tissues, it contains pre-adipocytes, which share some properties with macrophages. These cells express pattern recognition receptors (PRRs) and mature into macrophages, which contribute to local immune and inflammatory response.³⁹

To the best of our knowledge, this is the first study documenting the presence of herpes viruses in children with acute appendicitis using PCR in a large series of samples. Our results identified CMV and HHV-6 as pathogens to be pursued in the appendicitis patient groups, suggesting that viral infection or reactivation are associated with the disease. More studies are necessary to determine the underlying mechanisms and support the potential role of herpes viruses in the pathogenesis of acute appendicitis.

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Conflict of interest

We would like to declare that there is no financial or personal relationship with other people or organisations that could inappropriately influence our work during the submission process.

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