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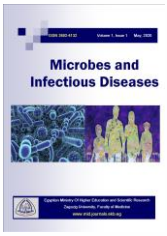

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Original article

Detection of cytomegalovirus (CMV) in fecal samples of cholestasis infants by real-time polymerase-chain reaction – a pilot study

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ABSTRACT

Background: Cholestasis is characterized by yellow symptoms of the skin, eyes, and mucous membranes. Many factors, such as biliary atresia, metabolic disease, and infection, can cause cholestasis. One of the leading causes of cholestasis is cytomegalovirus (CMV) infection. CMV detection is generally done through liver biopsy analysis, serological tests, and, most recently, polymerase-chain reaction (PCR) of body fluids such as blood, urine, and feces. Taking blood or tissue biopsies is invasive while taking urine from babies is difficult. This study aimed to assess the suitability of fecal samples as an alternative sample for detecting CMV in infants with non-biliary atresia cholestasis. **Method**: The design of this study was observational, with a cross-sectional approach. Clinical data on serological test results were collected from patient medical records. DNA was extracted from fecal samples, and CMV detection was performed using real-time PCR. **Results**: Four of thirteen (30.77%) patients showed active CMV infection through serological tests characterized by IgG+/IgM+. CMV infection was detected in 6/13 (46.15%) stool samples. The results showed that all patients who developed active CMV infection based on IgM serological testing had CMV DNA in their feces, with 66.67% sensitivity and 100% specificity. The relationship between the results of the anti-CMV serology test and fecal CMV PCR showed a strong correlation with a phi coefficient value of 0.720. **Conclusion**: The positive CMV PCR result on a fecal sample may be effective, help avoid invasive procedures such as liver biopsy and blood collection, and overcome the difficulties of urine sampling in infants.

Introduction

Cholestasis develops due to decreased bile formation or bile flow, and it might involve both extrahepatic and intrahepatic bile ducts or may be limited to one or the other [1, 2]. Infants afflicted with extrahepatic cholestasis generally do not exhibit symptoms of illness, possess completely acholic stools, and have an enlarged, firm liver, while patients with intrahepatic cholestasis frequently manifest signs of illness and fail to thrive. Histologic analysis of material taken during percutaneous liver biopsy can distinguish between extrahepatic and intrahepatic cholestasis [2]. The clinical manifestations of cholestatic liver disease may include pruritus, fatigue, jaundice, dark urine, and alcoholic feces [3]. However, jaundice is the

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most common symptom caused by indirect/ unconjugated or direct/unconjugated hyperbilirubinemia.

In neonates, jaundice typically occurs due to unconjugated hyperbilirubinemia, which is usually caused by increased red blood cell destruction, impaired hepatic uptake, and decreased bilirubin conjugation [4]. It commonly resolves spontaneously without intervention. Separating the serum bilirubin into conjugated (or direct) and unconjugated (or indirect) fractions is necessary to differentiate benign jaundice from cholestasis. Conjugated hyperbilirubinemia or cholestasis is often characterized by a conjugated or direct bilirubin level exceeding 1 mg/dL when the total bilirubin is below 5 mg/dL or more than 20% of the total bilirubin if the total bilirubin is above 5 mg/dL [5].

Based on a recent meta-analysis, cholestasis formed by several factors, including idiopathic neonatal hepatitis (26%), extrahepatic biliary atresia (26%), infection (12%), total parental nutrition (TPN)-associated cholestasis (6.5%), metabolic disease (4.37%), α1-antitrypsin deficiency (4.14%), and perinatal hypoxia/ischemia (4%). Cytomegalovirus (CMV) accounts for 33.5% of cholestasis cases caused by infection [6]. Cytomegalovirus (CMV) is known to cause intrahepatic bile duct damage. At the same time, the role of CMV in the development of extrahepatic cholestasis is not proven and is still a much-debated topic [7]. Detection of CMV in infants is often done through serological tests, which detect anti-CMV IgM and IgG. IgM assays have several weaknesses, such as low specificity towards primary infection due to false-positive outcomes, the potential persistence of IgM for several months after primary infection, and the possibility of positive results in reactivated CMV infections. Due to the limitations of the IgM tests, IgG avidity assays are conducted. A high level of anti-CMV IgG avidity indicates longstanding infection in an individual [8].

Recent CMV infection was also detectable with polymerase chain reaction (PCR) of a liver biopsy. This method has become the gold standard in the diagnosis of CMV infection followed by liver biopsy analysis. However, liver tissue biopsy is very invasive and rarely performed in infants [9-11]. Cytomegalovirus promptly activates its immediate-early (IE) genes after initial infection, which become detectable 2 to 4 hours after the virus enters the host cell. These genes generate two proteins, IE1-72 and IE1-86, crucial for virus replication and spreading throughout the body fluids [12]. CMV can be detected in body fluid samples, such as urine, blood, and feces [13]. Blood collection is an invasive technique that potentially causes inconvenience, and urine collection in infants is challenging. The perineal adhesive bag is the primary technique used for infant urine collection. However, it has elevated susceptibility to contamination and the potential for conducting false-positive results [14]. Stool sampling is non-invasive and easy to collect from infants. This pilot study was designed to evaluate the potential of fecal PCR CMV to aid the diagnosis of CMV infection in cholestasis infants and compare it with blood serologic testing.

Method

Sample Collection

This pilot study used a cross-sectional approach on thirteen cholestasis infants from dr. Cipto Mangunkusumo, the national referral hospital, central Jakarta. We add eleven age-matched healthy infants for additional data from non-symptomatic infants. The inclusion criteria for the cholestasis group in this study are infants with direct/conjugated bilirubin exceeding 1 mg/dL when total bilirubin is below 5 mg/dL or more than 20% of total bilirubin if total bilirubin is above 5 mg/dL and who undergo a CMV serological test. The exclusion criteria are cholestasis infants who receive antivirals or antibiotics. This analysis was approved by the ethics committee of the Cipto Mangunkusumo hospital and faculty of medicine, universitas Indonesia, with the number KET-1676/UN2.F1/LTIK/PPM.00.02/2023. Parental or legal guardian consent was obtained for every participant. Fecal samples were collected for a maximum of 1 hour after defecation. Feces were taken with a scope, placed in a sterile container, and stored at -80 C before analysis.

DNA extraction and quantification

Genomic DNA was isolated from 300 mg fecal samples using a QIAamp Fast DNA Stool Mini Kit (51604, QIAGEN, Germany). A nanodrop spectrophotometer 2000 (Thermo Scientific, MA, USA) was applied to quantify DNA. All DNA samples were kept at -20°C pending the execution of the Real-time PCR.

Primer and Probe

Real-time PCR was performed in the clinical microbiology laboratory, faculty of

medicine, universitas Indonesia, following their CMV diagnostic standard operating procedure accredited by ISO 15189. Briefly, RT-PCR was done using a lightcycler LC 96 machine (Roche, Germany) or a real-Time quantitative thermal cycler MA-6000 (Molarray, Suzhou, China). The PCR master mix was prepared by combining 10 µL of SensiFAST Probe No-ROX Mix (BIO-84005, Bioline, London, United Kingdom), 0.5 µL of CMV primer mix (Macrogen, South Korean), 1 µL of Probe CMV FAM-BHQ1 (Macrogen, South Korean), and 3.5 µL of nuclease-free water. The forward primer sequence was CATGAAGGTCTTTGCCCAGTAC, while the reverse sequence was GGCCAAAGTGTAGGCTACAATAG. The Probe CMV FAM-BHQ1 sequence was TGGCCCGTAGGTCATCCACACTAGG. Subsequently, 5 µL of DNA was added to the master mix. The PCR reaction was run with a pre-denaturation temperature cycle of 95°C for 3 minutes, denaturation at 95°C for 10 seconds, and 56°C for 60 seconds. Real-time PCR was conducted for a total of 45 cycles.

Statistical analysis

The prevalence of CMV in infants with cholestasis was calculated based on the positivity of the IgG+/IgM+ serological test indicating active infection. The sensitivity and specificity of fecal CMV PCR were calculated using serological testing as the most common diagnostic method for CMV infection in Indonesia. The correlation between fecal CMV PCR and anti-CMV serological testing in infants with cholestasis was calculated using the Fisher's Exact test. If the p-value <0.05, the results are interpreted as statistically different. The Phi Coefficient Value measured the strength of the relationship between fecal CMV PCR and anti-CMV serology tests. The maximum value of phi is 1, which indicates a strong association.

Result

Thirteen cholestasis infants were included in this study, and their demographic characteristics are shown in Table 1. All cholestasis patients showed an average age of 75.77±23.55 days old, with six females and seven males. The average total bilirubin level at sampling was 11.81±8.07 mg/dL, with a direct bilirubin level of 8.70±6.03 mg/dL. Liver injury markers such as gamma-glutamyl transferase (GGT), alkaline phosphatase (ALP), sspartate transaminase (AST), and alanine aminotransferase (ALT) were measured utilizing 575.01 ± 651.31, 539.77 ± 204.45, 284.39 ± 165.24, and 235.91±143.71 U/L.

The results of anti-CMV serological tests and PCR CMV of fecal samples in thirteen cholestatic infants are shown in Table 2. Immunoglobulin G was detected in 13/13 (100%) patients, while immunoglobulin M was detected in 4/13 (30.77%) patients. Acute or active CMV infection characterized by IgG+/IgM+ was detected in patients 1, 4, 5, and 8. In contrast, IgG+/IgM-, which indicates no longer active infection, was detected in the rest of the patients.

Detection of CMV samples in feces showed positive results in 6 (46.15%) patients and negative results in 7 (53.85%) cholestasis infants. The amplification curve is shown in Figure 1, showing six amplified samples with different Cq values from cholestasis infants. We tested 11 samples from healthy age-matched infants, resulting in 2 CMV PCR-positive cases without any sign of infection. CMV PCR data from blood samples was obtained from the medical records of patients 1, 3, and 9, while PCR CMV from urine samples was obtained from patient 7. The remaining patients underwent CMV PCR, but the samples used were unclear or did not undergo CMV PCR at all. Two patients had results that agreed between CMV PCR of blood and stool samples, while the rest showed different results. The CMV PCR results from the urine sample in patient 7 were consistent with the CMV PCR results from the fecal sample.

The results showed a significant correlation between the anti-CMV serological test and fecal CMV PCR with a value of $p = 0.021$ ($p<0.05$). The Phi coefficient shows a strong correlation between the anti-CMV serology test and fecal CMV PCR with a Phi value of 0.720. The sensitivity of the fecal CMV PCR test to the IgG serological test showed a figure of 100%. Meanwhile, the sensitivity and specificity test for fecal CMV PCR against IgM showed 66.67% sensitivity and 100% specificity. All cholestasis infants with anti-CMV IgG+/IgM+ serological testing had positive fecal CMV PCR results. In contrast, two cholestasis infants with anti-CMV IgG+/IgM- showed positive results for fecal PCR CMV.

Table 1. Characteristics of neonatal cholestasis

Characteristics	
Age (days ± SD)	75.77± 23.55
Sex (n)	
Male	7 (53.8%)
Female	6 (46.2%)
Clinical parameter	
Total Bilirubin (mg/dL ± SD)	11.81 ± 8.07
Direct Bilirubin (mg/dL ± SD)	8.70 ± 6.03
Gamma-Glutamyl Transferase (U/L ± SD)	575.01 ± 651.31
Alkaline Phosphatase (U/L ± SD)	539.77 ± 204.45
Aspartate Transaminase (U/L ± SD)	284.39 ± 165.24
Alanine Aminotransferase (U/L ± SD)	235.91 ± 143.71

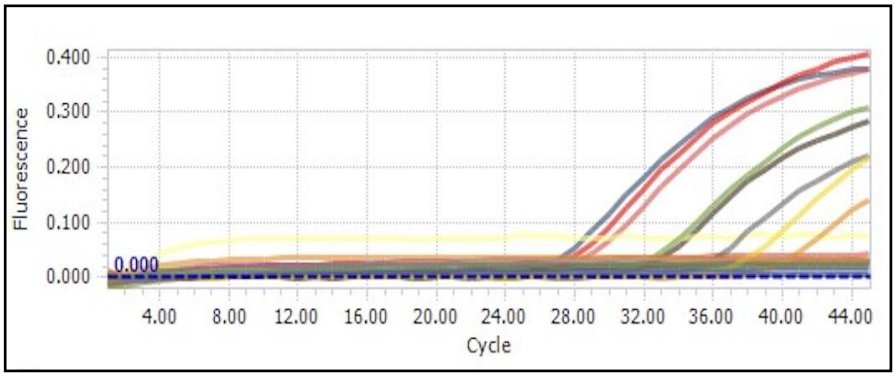
Table 2. Serology and PCR results for detecting CMV

Sample number	**IgG/IgM**	**PCR feces**
1	+/+	+
2	+/-	-
3	+/-	+
4	+/+	+
5	+/+	+
6	+/-	-
7	+/-	-
8	+/+	+
9	+/-	-
10	+/-	-
11	+/-	+
12	+/-	-
13	+/-	-

Table 3. Correlation between anti-CMV serology and stool CMV PCR

Serology Anti-CMV	**Faeces**			**P-value**
	Positive	**Negative**	**Total**	
IgG+/IgM+	4	0	4	0.021*
IgG+/IgM-	2	7	9	
Total	6	7	13	
Phi Coefficient	0.720			

Figure 1. Results of CMV DNA amplification plot from cholestasis



Discussion

Cholestasis can be formed by many factors, such as intrahepatic/extrahepatic bile duct obstruction and hepatocyte abnormalities caused by CMV infection, metabolic disorders, and hepatitis [6]. Management of cholestasis can vary depending on the cause. Therefore, an inaccurate diagnosis has the potential to result in inappropriate treatment and cause serious complications that can lead to death. The effective method for detecting CMV infection in cholestasis is through liver biopsy analysis, including PCR tissue and histological analysis [9, 10]. Even though it shows accurate detection results, liver biopsy is an invasive procedure and is rarely attempted in neonatal cholestasis [11]. Major and minor complications can occur in children after a liver biopsy. Minor complications appear as pain, subcapsular hemorrhage that does not require transfusion or prolonged hospitalization, infection, mild bile leak or hemobilia, and arteriovenous fistula. Major complications appear in the form of bleeding, pneumothorax or hemothorax, and even death [15]. Chaudhry et al. report 6.9% of complications after liver biopsy in infants, including bleeding and perihepatic ascites [16]. Biopsy was not performed in this study because it is invasive and difficult to obtain family consent. The alternative methods for CMV diagnosis are anti-CMV serology tests, antigenemia, cell-mediated immunization (CMI) assay, and PCR CMV from various body fluids such as blood and urine [9]. However, blood collection is also invasive and causes discomfort, while collecting baby urine is difficult. Alternative samples and detection methods may be useful to diagnose CMV infection in cholestasis infants.

Detection of CMV with analysis of liver biopsies in cholestasis, which is related to liver infection, has been shown as the most effective method because detection is carried out directly in the organs related to the disease [10]. In Indonesia, CMV detection in cholestasis infants is generally done through anti-CMV serological tests and blood/urine CMV PCR. The diagnosis of CMV infection in this study was confirmed through positive CMV IgG and IgM serology and/or positive CMV blood and urine via PCR. However, the origin of the samples from the PCR data we obtained needs to be clarified. Goel et al. showed that CMV IgM serological examination had a sensitivity of 69% and specificity of 61%. In comparison, blood CMV PCR had 61% sensitivity and 71% specificity associated with liver biopsy PCR [11].

In Indonesia, the agreement between CMV IgG and IgM compared to CMV PCR of liver tissue was weak and not in agreement with kappa values of 0.25 and 0.00 [17]. De Tommaso et al. investigated the diagnostic efficacy of serological examination with PCR analysis of liver and porta hepatis in the context of extrahepatic neonatal cholestasis. The result showed that serological examination had low accuracy (59%) in detecting active CMV infection, with 54% sensitivity and 61% specificity [18]. Even though some studies conclude that serological status has limited value compared to liver biopsy PCR CMV, this method is still the most widely used method to detect CMV infection. Based on serological tests, our results showed that 30.77% of cholestasis infants developed acute or active CMV infection, while the rest showed inactive CMV infection.

Detecting CMV by PCR of body fluids seems more efficient than serological status. PCR is a susceptible method for detecting CMV DNA because DNA fragment amplification still works despite the very low viral load [19]. Goel et al. had shown that blood PCR CMV had 61% sensitivity and 71% specificity compared to liver biopsy PCR CMV [11]. Another study reported urine PCR's role

in diagnosing congenital CMV with sensitivity results of 93% and 100% [20, 21]. Urine has long been used to detect congenital CMV in infants within the first three weeks of age [22]. Mastutik et al. showed that CMV DNA was positive in 87.2% of total extracted urine specimens in cholestasis infants. This study also showed that CMV detection in extracted urine was better than in unextracted urine, which only showed 48.8 % positivity [23]. These results suggested that PCR CMV for body fluid specimens is a promising method for detecting CMV infection. Due to the invasive method of blood collection and the difficulty of urine collection in infants, the fecal sample might be useful as an alternative. This study reports the presence of CMV in fecal samples of cholestasis infants and demonstrates the efficacy of the fecal specimen related to other body fluids.

The detection of CMV in stool samples from several diseases has been investigated for a long time. The study conducted by Herfarth et al. investigated the efficacy of CMV detection in stool samples from inflammatory bowel disease (IBD) patients. The study concluded that fecal PCR CMV showed higher accuracy than PCR CMV of tissue biopsy, with 83% sensitivity, 93% specificity, and 90% accuracy [24]. Ganzenmueller et al. investigated the diagnostic quantitative PCR CMV efficacy using stool samples in CMV intestinal disease. Results showed that PCR CMV had 67% sensitivity and 96% specificity in relation to PCR CMV of tissue biopsy [25]. However, CMV detection via PCR of stool samples has only been carried out in diseases related to the digestive organs. In our study, we investigated CMV detection in cholestasis liver disease.

Feces are body fluids that are related to the digestive system. Feces contain the gut microbiome, which is known to influence liver-related diseases such as nonalcoholic liver disease (NAFLD), alcoholic liver disease (ALD), biliary atresia, primary sclerosing cholangitis, hepatocellular carcinoma, cirrhosis, and all forms of cholestatic liver disease [26-28]. The gut-liver axis is a bidirectional interaction between the liver and intestines via the portal vein and bile duct. This interaction allows infectious agents in the intestine and liver to translocate from these two organs to each other [26]. This mechanism indicates the possibility of a direct link between CMV infection in the liver and the digestive tract in cholestasis sufferers. The question remains whether the presence of CMV in the gastrointestinal tract of cholestasis infants also indicates an infection in the gastrointestinal tract. Infections of the GIT tract can occur in the esophagus, stomach, small intestine, and colon. Although there are asymptomatic CMV infections, gastrointestinal tract CMV infections can be fatal [29].

This pilot study observed a high prevalence of CMV IgG in 100% (13/13) of samples. According to the study, the positive result of IgG in infants might result from persistent maternal antibodies or previous infection of CMV [30, 31]. Congenital CMV can differ from acquired infection in the first three weeks of life [7]. In our study, CMV detection was performed after two months of age, so the congenital infection is not likely confirmed. We also observed a 30.77% (4/13) prevalence of IgM-positive. IgM antibodies were produced immediately after viral infection, and their level is aligned with the viral DNA/RNA concentration in the body [23, 32]. Clearance of viral DNA usually results in the inactivation of IgM [23]. Based on serological tests, the infection is no longer active in 69.23% (9/13) of patients.

Interestingly, we found infants with serologically IgG+ and IgM-, which indicates previous CMV infection, but CMV DNA was found in fecal samples. The possible explanation for this phenomenon is that the CMV might be in the latent stages. As we know, CMV can establish lifelong latency after initial infection [33]. Unfortunately, we did not perform quantitative PCR, which could differentiate active from latent infection [34]. In addition, the result might indicate the early stages of reactivation, so the IgM has yet to be produced, but viral DNA has already existed. IgM antibodies may not be detected in peripheral blood when the patient is still in the early stages of the disease [35]. The reactivation of latent viruses is thought to be mediated by changes in post-translational histone modifications around the viral major immediate early promoter (MIEP), which expresses the viral major immediate early (IE) genes, resulting in full reactivation [36]. However, the reliability of fecal samples PCR CMV should be confirmed by comparing it with liver biopsy analysis.

This pilot study also found different results for PCR CMV in blood and fecal samples. Patient number 3, with negative blood PCR CMV (data not shown), showed positive CMV DNA in fecal samples. Two samples also showed positive CMV

PCR of fecal samples with negative PCR CMV of body fluids (data not shown). However, the type of body fluids remains unclear. This result indicates that CMV in feces might promote longer shedding than other body fluids. Puhakka et al. showed that shedding CMV in urine was still found at 18 months, which is more persistent than shedding in saliva and plasma [37]. Shedding CMV in fecal is intermittent and related to disseminated cytomegalic inclusion disease and viremia [38]. Although the use of stool samples appears promising, this study does not describe examinations using liver biopsy samples as the gold standard.

Our study showed that CMV detection in fecal samples was positive in 46.15% (6/13) of samples. Patients with positive fecal PCR CMV correlate better with serological status, showing 66.67% sensitivity and 100% specificity. We also collected the result of PCR CMV in urine or blood specimens. Unfortunately, the data was incomplete. The CMV PCR results from blood samples in patients number 1 and 9 were in line with the CMV PCR results from fecal samples. In patient 7, the result of the PCR CMV of the urine sample and the fecal sample's PCR CMV was align. However, the difference between blood and fecal PCR CMV results in patient 3 might indicate a false positive result. The positive result in healthy infants can not classified as congenital infection since the PCR was performed after three weeks of age, and there is no supplement data to analyze. The possible explanation is that CMV infection is asymptomatic, the extent of which requires screening for hearing loss and neurodevelopmental impairment as prevention [39]. We found a correlation between PCR CMV of fecal samples and CMV serology tests, with a significance value of 0.021. In addition, the phi coefficient value showed a strong correlation between PCR CMV of fecal samples and CMV serology tests with a value of 0.720. These numbers indicate that fecal CMV PCR accurately detects CMV infection and might be used as alternative body fluids for confirmed CMV infection after serological testing.

Conclusion

This pilot study found a strong association between serological tests and PCR CMV of fecal samples from cholestasis infants. Due to data limitations, the association between PCR CMV of fecal samples and PCR CMV of urine or blood samples could not be appropriately measured. Interim results indicate that CMV PCR of fecal samples might be helpful as an alternative body fluid to detect CMV. However, further research using a larger sample size is needed to ensure the applicability of using fecal samples as an alternative sample for diagnosing CMV infection. In future research, a correlation test between PCR CMV from liver tissue, blood, and urine samples and PCR CMV of fecal samples may be carried out.

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Conflict of interest

None declared.

Funding

None declared.

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