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ABSTRACT:

Cholestatic infants are associated with congenital abnormalities or viral infections, such as cytomegalovirus (CMV) infection. CMV can be detected by polymerase chain reaction (PCR) in body fluids, including urine which can be obtained easily and is non-invasive. The objective was to detect CMV in urine specimens of cholestasis infants and to analyze its correlation with serological status. This was a descriptive observational study with the cross-sectional approach, used urine from 39 cholestatic infants who meet the inclusion and exclusion criteria and have been approved by Ethics Committee. The nested-PCR was performed from extracted urine and unextracted direct urine. Serological data of immunoglobulin (Ig) M and IgG data were collected. Data were analyzed by Chi-square. Detection of CMV from extracted urine by PCR showed positive in 87.2% patients and from unextracted urine was positive in 48.7% patients. Serological status showed that IgM was positive in 41.0% patients and IgG was positive in 89.7% patients. The acute infection (IgM+ IgG+) was found in 41.0% patients, past infection (IgM-IgG+) was 48.7% patients, and not infected (IgM-IgG-) was in 10.3% patients. The acute infection (IgM+ IgG+), past infection (IgM-IgG+) and not infected (IgM-IgG-) was found in 41.0%, 48.7%, and 10.3% patients, respectively. The correlation between PCR CMV from extracted urine with serological CMV was moderate, while the unextracted urine was low. It indicates that to detect the infection of CMV, PCR technique is more accurate than serological testing, and the extracted urine is more appropriate specimen as PCR template than direct urine.

KEYWORDS: Infant mortality, Infectious disease, Cytomegalovirus, Polymerase chain reaction.

INTRODUCTION:

Cholestasis is an important cause of morbidity and mortality in infants and children[1-6]. Cholestasis is defined as decreased bile flow due to failure of secretion by hepatocytes or obstruction of bile flow to the intra or extra hepatic bile ducts.

The diagnosis of cholestasis is performed based on the examination of conjugated bilirubin levels, namely direct or conjugated bilirubin levels greater than 2 mg/dL if total bilirubin is less than 5 mg/dL, or more than 20% of total bilirubin if the bilirubin level is more than 5 mg/Dl[1-4,7]. The inability to detect and monitor the progression of liver damage will interfere the management of disease with cholestasis. The bile acids accumulation has an impact on hepatotoxicity so that it is an underlying cause of abnormalities in the liver[8-10]. If the cholestatic condition is not detected in early or not received the proper therapy, it will cause serious abnormalities that lead to death.

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The incidence of cholestasis in infancy is associated with congenital abnormalities or viral infection. The most common cause of infection is cytomegalovirus (CMV) infection[11] with symptoms of jaundice (62%), petechiae (58%), and hepatosplenomegaly (50%)[12]. Diagnosis of CMV infection in infants is carried out by isolating the virus from urine with culture techniques, blood serology by checking the detection of anti-CMV IgM and IgM, detection of CMV antigen by antigenemia assay on blood samples, detecting CMV DNA using polymerase chain reaction (PCR) techniques[13] and in situ hybridization in tissues[14]. Viral culture is the gold standard for the diagnosis of CMV. The virus was inoculated on fibroblast cells and observations were made from 2 to 21 days after virus inoculation[15]. In addition, false-negative results are often obtained when viral load is low. Currently in Indonesia, the most common diagnosis of CMV infection is by serological examination of CMV IgM and IgG from blood samples. IgM is formed 2-4 weeks after infection and IgG is produced following it[16]. However, in newborns, the ability to produce IgM and IgG is still weak. IgG for newborns is obtained maternally from the mother, while IgM is still in the learning process to synthesize slowly[17], so that the serological examination of IgM and IgG anti-CMV in infants in the first week is still not able to reflect the condition of CMV infection in infants[15].

The replication process of the virus initiated with the immediate-early (IE) gene expression in 2-4 hours after infection, then virions have been produced and spread throughout body fluids within 48-72 hours post-infection[18,19]. CMV DNA can be isolated from body tissues and body fluids such as tears, salivary glands, breast milk, urine, feces, semen, cervical secretions, amniotic fluid, blood, and organ transplants[20,21].

The CMV detection in the most conserve target areas, namely the major immediate early (MIE) gene and the late antigen (L) gene, have high specificity for detecting CMV DNA. Based on this, it is possible to detect CMV DNA from urine from the second- or third-day post-infection. The early and properly diagnosis will enable the patient to receive appropriate treatment so that it can reduce the mortality due to cholestasis.

Urine is a body fluid that is easy to obtain and non-invasive in newborns. The virus is excreted into the urine and mixes with cell debris along the urinary tract. For diagnostic purposes, it is necessary to consider whether the extracted or unextracted procedure of urine specimens is required to perform CMV PCR because eliminating one step means reducing costs and minimizing the time required. The objective of this study was to detect CMV in urine by PCR and to analyze the correlation between PCR CMV from extracted or unextracted urine (direct urine) specimens with the serological status anti-CMV in infants with cholestasis.

MATERIAL AND METHODS:

Sample Collection:

This study was a descriptive observational study with a cross-sectional approach. Samples were urine specimens from 39 infants with cholestasis in the Inpatient Unit of Children's Health at Dr. Soetomo General Academic Hospital Surabaya. Ethical study was approved by Ethics Commission, number 729/Panke.KKE /XII/2017. Subjects have been willing to participate in this study and have obtained consent from their parents or guardians by signing a written consent letter after receiving an informed consent explanation.

Inclusion criteria were 1-12 months infants with cholestasis who showed symptoms of jaundice in the following conditions: if total bilirubin is ≥5mg/dL, conjugated bilirubin level is 20% of total bilirubin levels, and if total bilirubin is <5mg/dL, the direct bilirubin level is >2mg/dL. Exclusion criteria were patients who had received antiviral therapy, malnourished patients, miliary tuberculosis patients, HIV patients, history of using immunosuppressive drugs such as corticosteroids and cytostatic, platelets <80,000mg/dL, ascites, and prolonged physiological hemostasis.

Serological Data collection:

The IgM and IgG anti-CMV were obtained by measuring IgM and IgG level using The VIDAS CMV IgM and IgG Assay (Biomerieux). IgM interpretation is the IgM index unit <0.7 negative, <0.7 - 0.9 equivocal, >0.9 positive. IgG interpretation is the IgG index unit <4 negative, >4 - <6 equivocal, >6 positive. This data were the secondary data from patient medical records.

PCR Data Collection:

The urine sample was divided into two parts, one part was directly used PCR without being extracted and the other part was extracted then using as PCR template. A total of 200µl of urine was used for CMV DNA extraction using the QIAamp DNA Mini Kit (Qiagen), the CMV DNA extraction procedure was carried out according to the protocol in the kit. The urine used by PCR was 4µl.

CMV DNA detection was performed by nested PCR technique using MIE4/MIE5 primers in the first round, then followed by IE1/IE2 primers in the second round. The MIE4 sequences were 5'-CCAAGC GGCCTC TGATAA CCAAGCC-3' and MIE5 sequences were 5'-CAGCAC CATCCT CCTCTT CCTCTGG-3' which produced 435 base pairs (bp). The IE1 sequences were5'-CCACCC GTGGTG CCAGCTCC-3' and IE2 were 5'CCCGCT CCTCCT GAGGACCC-3' which produced

161 bp[11]. PCR was performed using Go Taq Green Master Mix (Promega). The first PCR was conducted for the β-globin gene using PCO3+ and PCO4+ primers, subsequently proceed with PCR for CMV. The master mix reaction in the first round was 10µl master mix, 1 µl forward MIE4 10 pmol primer, 1µl 10 pmol MIE5 reverse primer, 4µl ddH2O, and 4µl template DNA (extracted or unextracted urine). The PCR conditions were pre-denaturation of 94°C for 5 minutes, denaturation of 94°C for 30 seconds, annealing 55°C for 30 seconds, extension at 72°C for 45 seconds, and PCR was performed for 40 cycles. Final extension 72°C was for 7 minutes. The master mix reaction and conditions for second round PCR were the same as the first round PCR, but the template was 1 µl of product from the first round PCR and used IE1 / IE2 primers. PCR results were electrophoretic on 2% acrylamide gel.

Statistical Analysis:

The correlation between the PCR of CMV DNA from the extracted and unextracted urine with serological anti-CMV in infants with cholestasis was performed by Chi-square. If significantly different, the strength of the relationship is known from the contingency coefficient.

RESULTS:

This study involved 39 infants with cholestasis consisting of 17 male infants and 22 female infants, aged 1-12 months (mean±SD= 3.44±2.326). Patients consisted of 32 patients aged 2 over 5 months. The level of direct/conjugated bilirubin (D Bil) was 8.1221±4.15911 (mean±SD) and total bilirubin (T Bil) was 11.1603± 5.54247 (mean±SD) (Table 1).

Table 1. Characteristics of infants with cholestasis

Characteristics	
Age (mean ± SD)	3,44 ± 2,326
Age (N / %)	
1-2 month	18 (46.1 %)
3-4 month	14 (35.9%)
5-6 month	3 (7.7%)
>6 month	4 (10.3%)
Sex (N / %)	
Male	22 (56.4%)
Female	17 (43.6%)
Bilirubin index (mean ± SD)	
Direct Bilirubin	8.1221 ± 4.15911
Total Bilirubin	11.1603 ± 5.54247
Type Cholestasis N (%)	
Intrahepatic	23 (59%)
Extrahepatic	16 (41%)
SD: Standard Deviation; N: Number	

The results of serological data showed that 16/39 (41.0%) patients were IgM positive and 35/39 (89.7%) patients were IgG positive. The results of serological examinations showed acute CMV infection indicated by IgM+ IgG+ was 16/39 (41.0%), had been infected CMV indicated by IgM- IgG+ was 19/39 (48.7%), and not infected CMV indicated by the IgM- IgG- was 4/39 (10.3%) (Table 2).

Table 2. Detection of CMV infection based on serology and PCR from urine

Examination type		Number	Percentage
PCR extracted urine	Positive	34	87,2%
	Negative	5	12,8%
PCR unextracted urine	Positive	19	48,7%
	Negative	20	51,3%
Serological	IgM+	16	41.0%
	IgG+	35	89.7%
	IgM+, IgG+	16	41.0%
	IgM-, IgG+	19	48.7%
	IgM-, IgG-	4	10.3%

Detection of CMV infection using conventional PCR techniques from extracted urine showed 34 (87.2%) patients were positive and 5 (12.8%) were negative, and from unextracted urine showed 19 (48.7%) patients were positive and 20 (51.3%) patients were negative (Figure 1, Table 2).

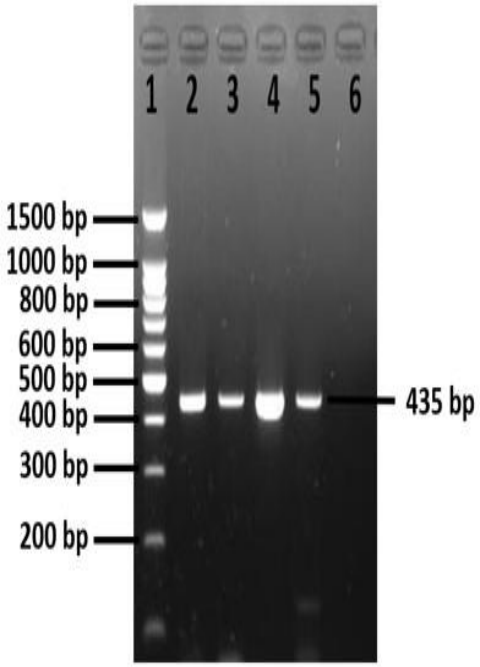

Figure 1. Detection of CMV infection by polymerase chain reaction (PCR) using MIE4/MIE5 primers from urine specimens. Lane 1= Marker 100 base pair, lane 2, 3, 4, 5 = specimens showed positive (± 435 base pair), lane 6 = negative control.

There was no difference between PCR CMV from the extracted and unextracted urine (p=0.064). However, these data suggest that the PCR of unextracted urine with positive results, also showed the same positive results with the PCR of extracted urine. In addition, the PCR of unextracted urine with negative results, showed 75% positive and 25% negative in the PCR of extracted urine (Table 3).

Table 3. The PCR results from the extracted and unextracted urine in infants with cholestasis

The PCR from unextracted urine	The PCR from extracted urine		Total	P value
	Positive	Negative		
Positive	19 (100%)	0 (0%)	19 (100%)	0,064
Negative	15 (75%)	5 (25%)	20 (100%)	
Total	34 (87.2%)	5 (12.8%)	39 (100%)	

There was a correlation between anti-CMV serological examination and PCR examination of extracted urine with p value = 0.028 (p <0.05). Contingency coefficient was significantly different with a value of 0.4 (p = 0.024) and showed moderate correlation strength (Table 4). All infants with cholestasis who presented the IgM+ IgG+ had positive for CMV PCR results. When IgM and IgG were negative, but the urine PCR results were positive.

There was a correlation between anti-CMV serological examination and PCR examination of unextracted urine (p = 0.035). The contingency coefficient was 0.383, which was significantly different (p = 0.035) and showed low association strength (Table 4). These results suggested that in patients who are serologically negative for IgM and IgG, CMV DNA is also absent in the urine specimen.

Table 4. Correlation between anti-CMV serology and PCR of extracted and unextracted urine in infants with cholestasis

Serological anti-CMV	The PCR from extracted urine		Total	P-Value
	Positive	Negative		
IgM+, IgG+	16 (100.0%)	0	16 (100.0%)	0.028*
IgM-, IgG+	16 (84.2%)	3 (15.8%)	19 (100.0%)	
IgM-, IgG-	2 (50.0%)	2 (50.0%)	4 (100.0%)	
Total	34 (87.2%)	5 (12.8%)	39 (100.0%)	
	Coefficient contingency of the PCR from extracted urine were 0.4 (p = 0.024)			
	The PCR from unextracted urine		Total	P-Value
IgM+, IgG+	11 (68.8%)	5 (31.3%)	16 (100.0%)	0.035*
IgM-, IgG+	8 (42.1%)	11 (57.9%)	19 (100.0%)	
IgM-, IgG-	0 (0%)	4 (100.0%)	4 (100.0%)	
Total	19 (48.7%)	20 (51.3%)	39 (100.0%)	
	Coefficient contingency of the PCR from unextracted urine were 0.383 (p = 0.035)			

DISCUSSION:

Cholestasis can be classified into extrahepatic cholestasis which is a mechanical obstruction of the extrahepatic bile duct and intrahepatic cholestasis caused by intrahepatic bile duct obstruction and the presence of hepatocyte abnormalities[22]. Symptoms of cholestasis are jaundice, pale stools, dark urine, enlarged liver, sometimes splenomegaly[23,24]. Infants with CMV infection will receive antiviral or immunomodulatory treatment[25]. Infants with cholestasis who do not receive proper treatment, usually their condition will get worse and cause death. Based on this, a quick diagnosis of CMV infection will be useful for reducing the mortality rate due to cholestasis.

Currently, the most widely used method to diagnose CMV infection is serological anti-CMV. This method can detect the presence of specific anti-CMV IgM and IgG antibodies in the circulation among infants with cholestasis[11] and among pregnant women[26]. In addition, the detection of IgM and IgG is also useful in determining whether the patient has CMV infection acutely or has CMV infection in the past. The indication of acute or recent infection is positive for IgM and positive or negative for IgG, while the past infection is positive for IgG and negative for IgM[15]. Based on serological CMV, it showed that 41.0% of infants with cholestasis were in acute CMV infection and 48.7% of infants had been infected with CMV. Furthermore, 10.3% infants were negative for CMV infection based on serological examination.

CMV is an important viral infection in neonatal cholestasis[27,28] and the reason of congenital infection[29]. This study demonstrated that antibodies to CMV were high, whereas CMV IgM was 41% and CMV IgG was 89.7%. Other studies showed that CMV IgM in neonatal cholestasis was 30-40% and 80-90% had IgG (and/or IgM) CMV in serum[27,30]. The infants age who positive for IgM aged 2, 3, 4, and 5 months, and who positive for IgG aged 1-2 months were 15 infants and 29 infants were more than 2 months. Seropositivity of CMV IgM or IgG in this study cannot confirm whether CMV infection is congenital or peri-natal or post-natal. In addition, seropositivity of CMV IgG also cannot be determined whether obtained from maternal or not. Because congenital infection of CMV can be determined and distinguished from acquired infection in perinatal or post-natal infection certainly before third week of life[15]. In this study, identification CMV was performed in infants after 4 weeks ages, therefore it is not possible to confirm the congenital infection.

PCR examination is a method to detect DNA that is useful in diagnosing viral diseases because of the ability to detect viral DNA even though the viral load is low[31,32]. This is a sensitive technique for detecting CMV based on the viral nucleic acid amplification reaction. CMV can be detected from urine, blood, or saliva within

the first 3 weeks of life by viral culture or PCR[33]. Detection DNA CMV by PCR from urine specimens can increased sensitivity compared to viral culture from urine[15]. Viral culture was observed for 2 to 21 days to ensure the results were negative. It is expensive and laborious. PCR is being used more frequently for diagnosis of viral infections because of its enhanced sensitivity and rapid turn-around[15]. In addition, it can reduce the examination time from 3 weeks to a day, save costs, and be rapid.

This study demonstrated that CMV DNA in urine specimens of cholestatic infants was very high (87.2%). This study was in accordance with others that testing CMV by PCR using blood specimens from cholestasis infants that showed 74% positive[30]. This suggested that the infection of CMV is the most common of infection that causes cholestasis in infants[11], as reported in another study that showed CMV infection can play a role in the pathogenesis and progression of extrahepatic biliary atresia[34].

This study detected CMV DNA in two ways, namely by extracting DNA from urine and directly using urine as PCR template without DNA extraction step. The results showed that no difference in PCR results between using the extracted urine and unextracted urine as PCR template, but it seems that the extracted urine showed a better indication than the unextracted urine. It was 87.2% positive for the extracted urine and 48.7% for the unextracted urine. Moreover, all positive PCR results from the unextracted urine, also showed positive PCR results from the extracted urine. Meanwhile, the negative PCR results from unextracted urine, some of them still showed positive PCR results from the extracted urine. It is probably because the DNA molecule is bound to the silica-gel membrane during the DNA extraction process, while the protein and other contaminants molecules have dissolved in washing step. Therefore, PCR primers were annealed to the target genes properly, the amplification process could be maximized and showed positive PCR results.

This study found a moderate association between CMV serology and CMV PCR from the extracted urine specimens and a low association with the unextracted urine specimens in infants with cholestasis. It indicates that if the serologic result is positive then the possibility of a positive PCR result from the extracted urine is moderate. This can be seen in the results, if IgM+ IgG+, so all PCR results are positive, while IgM- IgG+ and IgM- IgG-, then the PCR results can be positive and can be negative. Furthermore, in PCR results from the unextracted urine, if the serologic result is positive, then the possibility of positive PCR results is low. This can be seen in the results, the IgM+ IgG+, IgM- IgG+, or IgM- IgG-, so the PCR results can be positive and can be negative.

IgM antibodies were produced immediately after viral exposure, accompanied by an increase of DNA CMV excretion and a gradual production of IgG antibodies. IgM to CMV appeared for 2 to 3 weeks post-infection and gradually decreased in post-acute infection, while IgG to CMV appeared 2 to 3 weeks primary infection and persist for life[16,30,35,36]. Cleansing of CMV DNA is correlated with clearance of IgM antibodies[37]. We found that the infants were IgM negative and IgG positive, while CMV DNA was positive. Infants aged 1-2 months were 9 infants, 3-4 months were 3 infants, and more than 4 months were 4 infants. The possible explanation is that the infants in this study could be post-acute infection, but still in the active period to produce CMV DNA, and IgG is still elevated and produced during life. In addition, we also found the infant with IgM was negative, IgG was positive, and DNA CMV was negative. There were 3 infants aged 2, 3 and 5 months. The possible explanation is that the IgG was from persistent maternal antibodies or maybe the infants have been already infected by CMV, so IgM must be no longer be produced, CMV DNA is cleared, and IgG still persists for long life[30,35,36].

Interestingly, we found infants who serologically IgM and IgG were negative but DNA CMV in urine was positive. The infants were 2 and 4 months. The explanation may be because CMV infection is still in its early stages so that IgM and IgG have not been appeared, and viral DNA has already been excreted. It can be an early onset of infection or the early time after incubation period so that IgM and IgG has not been produced. Infection of CMV is initiated by inoculation of the virus onto a mucosal site by hand contact or intimate contact. The incubation period is around one months. In primary infection, the viremia is present, and the virus is shed from multiple sites, including saliva, urine, cervicovaginal secretion, and semen. IgM and IgG appear in serum immediately after onset of viral shedding[38].

Infants who were serologically negative for IgM and IgG, viral DNA CMV was not found in the urine. The data showed that infants were 2 months. Infection of CMV is not always only factor causing cholestasis in infants. The most common causes of cholestasis in infants are idiopathic neonatal cholestasis, alpha-1 anti-trypsin deficiency, infections, and biliary atresia[22,39]. In the early stages of infection, the virus was excreted in very small amounts or IgM has not been formed, so it was not detected in serum and in urine. Overall, if the serological examination shows negative but the patient shows symptom of cholestasis in clinically appearance, it is suggested to confirm the diagnosis by PCR of the extracted urine.

In conclusion, this study found a moderate association between serological and PCR testing to detect CMV infection in infants with cholestasis from extracted urine and a low association from unextracted urine. It indicates that the PCR technique for detecting CMV infection is more accurate than serological testing to CMV, and the extracted urine is more appropriate specimen as a PCR template than direct urine.

CONFLICT OF INTEREST:

The authors declare no conflict of interest.

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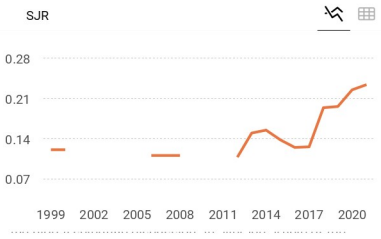

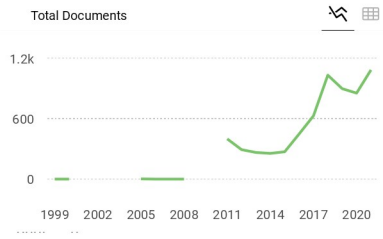

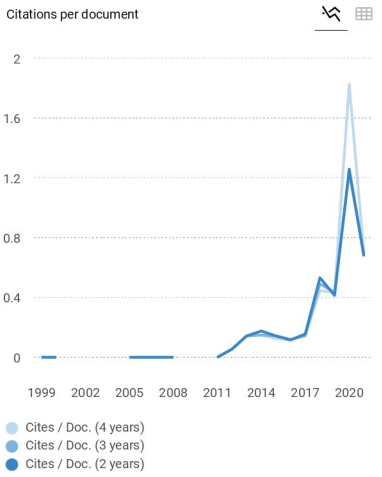

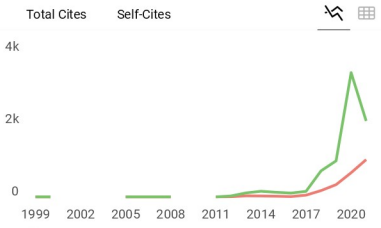

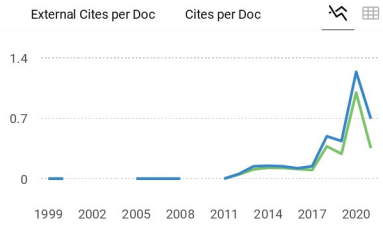

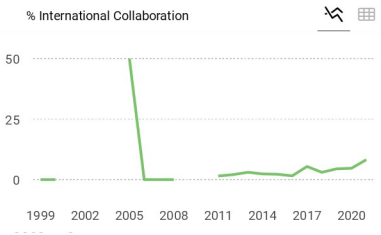

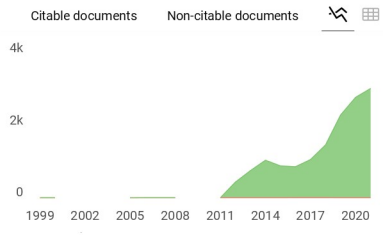

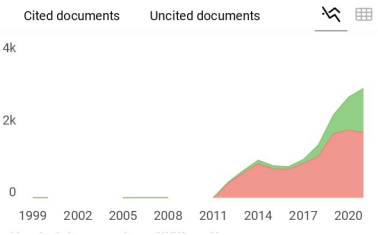

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