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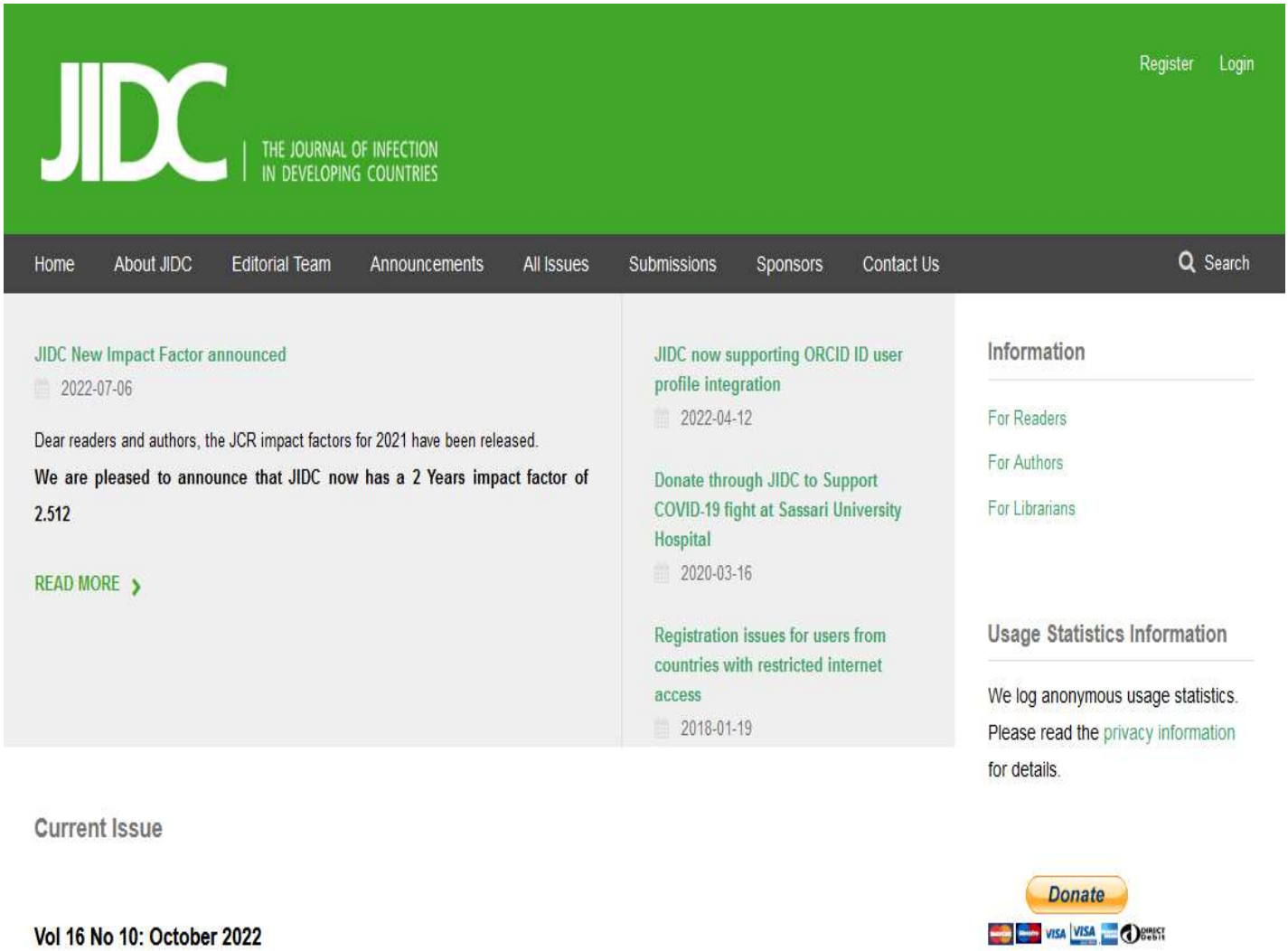

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*Original Article*

# Polymerase chain reaction of human cytomegalovirus from liver and urine compared with serological test in cholestasis infants

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## Abstract

Introduction: The most common infection in cholestatic infants is caused by human cytomegalovirus (HCMV). The aims were to detect the presentation of HCMV in cholestatic infants and to evaluate the concordance, sensitivity, and specificity between serology and polymerase chain reaction (PCR) of HCMV from liver biopsy and urine specimens.

Methodology: A descriptive observational study with a cross-sectional approach was conducted on 35 cholestatic infants with ethical approval. Specimens were liver biopsy, urine, and anti-HCMV serology. Liver and urine specimens were performed to nested PCR, followed by statistical analysis.

Results: PCR from the liver biopsy and urine specimen were positive in 74.3% and 85.7%, respectively. There was no concordance between IgM with the liver PCR, but there was a concordance between IgM with the urine PCR and between IgG with the liver and urine PCR. The sensitivity and specificity of IgM with the liver PCR were 46 % and 56%, respectively, with a diagnostic accuracy of 49%. While IgG sensitivity was 96% with a diagnostic accuracy of 80%. IgG sensitivity and IgM specificity compared with the urine PCR were 93% and 100%, respectively, with a diagnostic accuracy of more than 60%.

Conclusions: It demonstrates a high prevalence of HCMV DNA in urine and liver biopsy from cholestatic infants. HCMV PCR assay is more sensitive and specific than the anti-HCMV IgM, but IgG has high sensitivity and accuracy diagnostic. Therefore, serological examination is an option for diagnosing HCMV infection in cholestatic infants in developing countries with no PCR facilities.

**Key words:** Infant mortality; infectious disease; developing country; human cytomegalovirus.

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## Introduction

The infant mortality rate in developing countries is still high. One of the causes is cholestasis. The incidence of cholestasis in infants is associated with congenital abnormalities or viral infections. The most common cause of infection was Human cytomegalovirus (HCMV) infection [1]. Data showed that the seroprevalence of HCMV in women of childbearing age is approximately 40-80% in a developed country and 90-100% in developing countries [2]. Therefore, it causes the congenital transmission of the virus from mothers who are primary HCMV infected to the fetus [3,4]. This congenital infection of HCMV occurs in approximately 0.5-0.7% of live births [3,5,6]. Most infected newborns are asymptomatic [3,5], but approximately 11% of live birth with congenital HCMV infection were symptomatic [3] such as jaundice (62%), petechiae (58%), hepatosplenomegaly (50%) [1], and up to 20% develop sensorineural hearing loss or other permanent neurologic sequelae [5] and lead to permanent disabilities [3]. Therefore, proper early diagnosis is very important in order to provide appropriate therapy and reduce the occurrence of permanent disability.

Currently in Indonesia, the most frequently used method for diagnosing HCMV infection is a serological examination of anti-HCMV immunoglobulin M (IgM) and immunoglobulin (IgG) from blood samples. The presentation of IgM antibody to HCMV is formed approximately 1-2 weeks after infections, the titer peaks

in 1-3 months, then begins to decrease, and remains detectable up to 4 months [4,5,7]. In addition, anti-HCMV IgG can be detected 2-3 weeks after the appearance of symptoms [4,5] and maternally anti-HCMV IgG from the mother can be detected up to 8 months [2]. However, the sensitivity of IgM detection is still low where IgM was found to be negative in more than 50% of symptomatic children, while in asymptomatic children it was 78% [8]. Therefore, serological examination of anti-CMV IgM and IgG in newborns still cannot fully indicate the presence of HCMV infection in infants.

Polymerase chain reaction (PCR) examination is a virological detection method that is useful in diagnosing viral diseases because of its ability to detect very small amounts of viral DNA. HCMV DNA from infants can be isolated from body tissues such as liver biopsy tissues and body fluids such as tears, salivary, and urine [9,10]. The most common gene target area is the immediate early (IE) gene. On 2-4 hours after infection, the IE gene begins to activate the replication process, and intact virions spread in all body fluids within 48-72 hours after infection [11,12]. Therefore, the presentation of HCMV DNA can be detected from body fluids on the second or third day after infection. The objective of this study was to detect the presentation of HCMV DNA in the liver tissues and urine specimens from infants with cholestasis by PCR and to evaluate the concordance of the IgM and IgG anti-HCMV with PCR of HCMV from liver tissues and urine, as well as the sensitivity and specificity of serological test compared to PCR.

## Methodology

*Sample collection*

This study was a descriptive observational study with a cross-sectional approach. This study has received approval from the ethical commission with ethical clearance number 729/Panke.KKE/XII/2017. All parents or guardians of the subjects in this study have received an informed consent explanation and were willing to participate in this study.

The samples were 35 infants with cholestasis who were treated at the Pediatric Inpatient Installation, Department of Child Health, Dr. Soetomo General Academic Hospital Surabaya in the period December 2017 to December 2018. The operational definition of cholestasis in this study was infants with jaundice, where the conjugated bilirubin level is 20% of the total bilirubin level (if the total bilirubin is greater than (>) 5 milligrams per deciliter (mg/dL) or the direct bilirubin level is > 2 mg/dL (if the total bilirubin is less than (<) 5 mg/dL). Specimen taken from patients were liver biopsy, urine, and serological data. Inclusion criteria were infants with cholestasis and aged 1 to 6 months. Exclusion criteria were patients who had received antiviral therapy, HIV patients, miliary tuberculosis patients, malnourished patients, history of using immunosuppressive drugs such as corticosteroids and cytostatic, platelets < 80.000 mg/dL, prolonged hemostasis function, and ascites.

*Serological data collection*

Serological examination, which includes anti-HCMV IgM and IgG levels was examined by the Enzyme Linked Fluorescent Assay (ELISA) method using a solid phase receptacle from VIDAS. The interpretation of IgM was that IgM index unit < 0.7 was negative, < 0.7 to 0.9 was equivocal, > 0.9 was positive. The interpretation of IgG was that IgG index unit < 4 was negative, > 4 to < 6 was equivocal, and > 6 was positive.

*HCMV PCR from liver biopsy and urine specimens*

The liver biopsy and urine specimens were collected in a sterile collection tube and then taken to the Institute of Tropical Diseases, Airlangga University for identification of HCMV infection by nested PCR. Extraction was carried out using the QIAamp DNA Mini Kit (QIAGEN, Hilden, Germany) according to the protocol then followed by PCR using primer as reported previously [13,14].

The β globin gene PCR was performed using PCO3+ and PCO4+ primers and the PCR Mastermix (PROMEGA, Madison, USA) which product size were the 325 base pair (bp). The compositions were 10 µL master mix (Promega), 1 µL PCO3+ (in a concentration of 10 picomole), 1 µL PCO4+ (in a concentration of 10 picomole), 5 µL $ddH_2O$, 3 µl DNA template. The initial denaturation 5 minutes at 94 °C for 1 cycle, then 30 seconds of denaturation at 94 °C, 30 seconds of annealing at 55 °C, 45 seconds of elongation at 72 °C, for all were in 40 cycles, and then 7 minutes for final elongation at 72 °C.

The PCR of HCMV DNA was performed using the MIE4 and MIE5 primers which resulted in size 435 bp for the first round and the IE1 and IE2 primer for the second round which resulted in size 161 bp. The compositions were 10 µL master mix (Promega), 1 µL the forward primer, 1 µL the reverse primer, 4 µL $ddH_2O$, and 4 µL the DNA template. The PCR conditions were 5 minutes of initial denaturation at 94 °C, 30 seconds of denaturation at 94 °C, 30 seconds of annealing at 67 °C, and 45 seconds of elongation at 72

°C. All were carried out for 40 cycles, then 7 minutes for final elongation at 72 °C.

*Statistical Analysis*

The presentation of HCMV DNA in liver biopsy and urine specimens was shown in percentage. The concordance of anti-HCMV IgM and IgG with HCMV PCR from liver and urine specimens was analyzed by the Fisher's Exact Test 2-sided and McNemar. The sensitivity and specificity were shown in percentage.

*Ethical permission*

The ethical was obtained from the Dr. Soetomo General Academic Hospital, Surabaya, number 729/Panke.KKE/XII/ 2017.

## Results

There were 35 infants with cholestasis involved in this study, consisting of 20 males and 15 females aged between 1 to 6 months (mean ± SD = 2.771 ± 1.087). The levels of direct/conjugated bilirubin (D Bil) were 7.955 ± 4.674 (mean ± SD) and the total bilirubin (T Bil) was 10.369 ± 5.896 (mean ± SD) (Table 1).

All samples in this study showed positive results for PCR of the β globin gene, hence continued with detection of HCMV. The result of HCMV PCR from liver tissues and urine specimens were positive in 26/35 (74.3%) and in 30/35 (85.7%) infants, respectively. The product of HCMV PCR is shown in Figure 1.

**Table 1.** Patient Characteristics.

| Characteristics | |
|---|---|
| **Age** (mean ± SD) | 2.771 ± 1.087 |
| **Age** (N (%)) | |
| 1-2 month | 18 (51.4) |
| 3-4 month | 14 (40) |
| 5-6 month | 3 (8.6) |
| **Sex** (N (%)) | |
| Male | 20 (57.1) |
| Female | 15 (42.9) |
| **Bilirubin index** (mean ± SD) | |
| Direct Bilirubin | 7.955 ± 4.674 |
| Total Bilirubin | 10.369 ± 5.896 |

SD: Standard Deviation; N: Number.

Serological data showed that IgM positive were found in 16/35 (45.7%) infants and IgG positive were found in 31/35 (88.6%) infants. Acute infection (IgM+ and IgG+), past infection (IgM- and IgG+), and uninfected (IgM- and IgG-) were found in 16/35 (45.7%), 15/35 (42.9%), and 4/35 (11.4%) infants, respectively (Table 2).

There was no concordance between anti-HCMV IgM with HCMV PCR from liver biopsy ($p > 0.05$; $p = 0.929$), but there was concordance between anti-HCMV IgM with HCMV PCR from urine specimens ($p < 0.05$, $p = 0.027$) which kappa coefficient was 0.246 (fair: 0.21 – 0.4) (Table 3).

**Figure 1.** The β-globin gene PCR result size 110 base pair (bp) in lane 2, 3, 4, 5 (**A**) and the HCMV PCR result size 435 bp for first round in lane 2, 3, 4 and 161 bp for second round in lane 5, 6 (**B**). The line 1 is PCR marker.

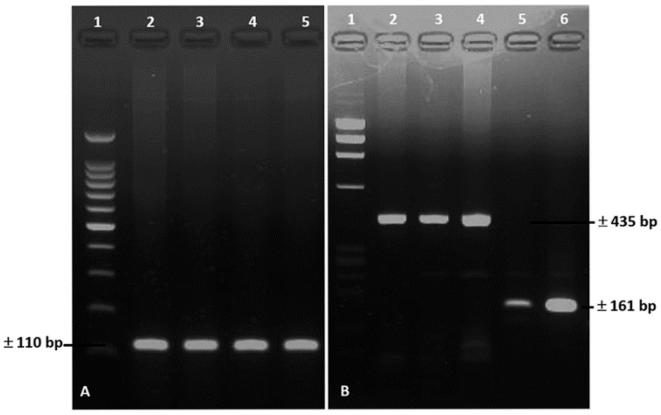


**Table 2.** HCMV PCR and anti-HCMV serological from infants with cholestasis.

| Characteristics | N (%) |
|---|---|
| **HCMV PCR from Liver** | |
| Positive | 26 (74.3) |
| Negative | 9 (25.7) |
| **HCMV PCR from Urine** | |
| Positive | 30 (85.7) |
| Negative | 5 (14.3) |
| **Serological anti-HCMV** | |
| IgM Positive | 16 (45.7) |
| IgM Negative | 19 (54.3) |
| IgG Positive | 31 (88.6) |
| IgG Negative | 4 (11.4) |
| IgM +, IgG + (acute infections) | 16 (45.7) |
| IgM -, IgG + (past infection) | 15 (42.9) |
| IgM -, IgG - (uninfected) | 4 (11.4) |

HCMV: human cytomegalovirus; PCR: polymerase chain reaction; IgM: immunoglobulin M; IgG: immunoglobulin G.

**Table 3.** The concordance of anti-HCMV IgM with HCMV PCR from liver biopsy and urine specimens.

| The specimens of HCMV PCR | IgM | | Total | Coefficient Kappa | *p* value |
|---|---|---|---|---|---|
| | Positive | Negative | | | |
| Liver - Positive | 12 (46.2%) | 14 (53.8%) | 26 (100%) | 0.013 | 0.929 |
| Liver - Negative | 4 (44.4%) | 5 (55.6%) | 9 (100%) | | |
| Urine - Positive | 16 (53.3%) | 14 (46.7%) | 30 (100%) | 0.246 | 0.027 |
| Urine - Negative | 0 (0%) | 5 (100%) | 5 (100%) | | |

HCMV: human cytomegalovirus; PCR: polymerase chain reaction; IgM: immunoglobulin M.

**Table 4.** The concordance of anti-HCMV IgG with HCMV PCR from liver biopsy and urine specimens.

| The specimens of HCMV PCR | IgG | | Total | Coefficient Kappa | *p* value |
|---|---|---|---|---|---|
| | Positive | Negative | | | |
| Liver - Positive | 25 (96.2%) | 1 (3.8%) | 26 (100%) | 0.360 | 0.017 |
| Liver - Negative | 6 (66.7%) | 3 (33.3%) | 9 (100%) | | |
| Urine - Positive | 28 (93.3%) | 2 (6.7%) | 30 (100%) | 0.364 | 0.030 |
| Urine - Negative | 3 (60%) | 2 (40%) | 5 (100%) | | |

HCMV: human cytomegalovirus; PCR: polymerase chain reaction; IgG: immunoglobulin G.

There was concordance between anti-HCMV IgG with HCMV PCR from liver biopsy ($p < 0.05$; $p = 0.017$) and from urine specimens ($p < 0.05$; $p = 0.030$) with kappa coefficient were 0.360 for HCMV PCR from liver biopsy and 0.364 for HCMV PCR from urine specimens (fair: 0.21 – 0.4) (Table 4).

McNemar (exact sig 2-sided) test showed that there was a significant difference between HCMV PCR from liver biopsy and urine specimens with anti-HCMV IgM ($p < 0.05$, liver: 0.031, urine: < 0.001), but there was no significant difference between HCMV PCR from liver biopsy and urine specimens with anti-HCMV IgG ($p > 0.05$, liver: 0.125, urine: 1.000) (Table 5).

The sensitivity and specificity of IgM anti-HCMV compared with HCMV PCR of liver biopsy specimens were 46.15% and 55.55%, respectively, with a diagnostic accuracy of 48.57%. While the sensitivity of anti-HCMV IgG is 96.15% with a diagnostic accuracy of 80%. In addition, the sensitivity of IgG and specificity of IgM compared to HCMV PCR of urine specimen showed 93.33% and 100%, respectively, with a diagnostic accuracy of more than 60% (Table 5).

## Discussion

Cholestasis is a decrease or obstruction of bile flow at any stage to the extrahepatic biliary tract and duodenum with the main symptoms of cholestasis are jaundice, acholic stools, and dark urine [15-17]. This condition is the most common cause of morbidity and mortality in infants and children. The accumulation of bile acids has an impact on hepatotoxicity. Therefore, it becomes the underlying cause of liver disorders [18]. The identity of prolonged neonatal jaundice more than 2 weeks of early life is an essential procedure for an early diagnosis of cholestasis diseases [10]. The inability to detect and monitor the progression of liver damage will hinder the appropriate management of cholestatic disease.

The most common causes of cholestasis are biliary atresia, α-1 antitrypsin deficiency, and infection, including HCMV infection [20]. HCMV can be transmitted horizontal or maternal from mother to fetus or infant [21]. In this study, the time of infection could not be determined whether during prenatal, natal or postnatal periods, due to the age of infants involved in this study was variable from 1 to 6 months, even though more than 50% of cholestasis occurred in 1 to 2 months infants. This requires further confirmation.

This study used specimens from liver biopsy and urine. It showed high prevalence of HCMV DNA in cholestatic infants. HCMV DNA detected in more than a half of patient that was 74.3% of liver tissue and 85.7% of urine. Another study in liver biopsy tissues in cholestasis infants showed that 48% [22], 34.3% [23], and 52% [24] patients were positive for HCMV DNA. In a Brazilian study on patients with extrahepatic cholestasis, of 33 liver biopsy samples examined by HCMV PCR, 27.3% were positive for HCMV DNA [23]. Research in Egypt involving 94 patients with biliary atresia and 91 patients with neonatal cholestasis due to other causes (non-biliary atresia), the frequency of HCMV DNA by PCR examination of liver biopsy in patients with biliary atresia was 5.3%, non-biliary atresia 23% [25]. In addition, PCR of urine samples was considered the optimal sample for the detection of HCMV infection in newborns. The PCR results showed that there were 79 of 80 (98.8%) positive urine samples [26].

The use of PCR as a diagnostic method in developing countries is not routinely carried out due to limited equipment and funds. Therefore, serological examination is still used as an alternative method for

**Table 5.** Sensitivity and Specificity of anti-HCMV serology compared with HCMV PCR from liver biopsy and urine specimens.

| Serology compared with HCMV PCR | Sensitivity | Specificity | PPV | NPV | DA | Mc Nemar |
|---|---|---|---|---|---|---|
| IgM compared with Liver | 46.15% | 55.55% | 75.00% | 26.31% | 48.57% | 0.031 |
| IgG compared with Liver | 96.15% | 33.33% | 71.42% | 75% | 80% | 0.125 |
| IgM compared with Urine | 53.33% | 100% | 100% | 26.31% | 60% | <0.001 |
| IgG compared with Urine | 93.33% | 40% | 90.32% | 50% | 85.71% | 1 |

HCMV: human cytomegalovirus; PCR: polymerase chain reaction; IgM: immunoglobulin M; IgG: immunoglobulin G; PPV: positive predictive value; NPV: negative predictive value; DA: diagnostic accuracy

diagnosing HCMV infection. Serological tests are very useful to determine infection condition, acute infection, or recent infection by examining the IgM or in past infections by examining the presence of HCMV IgG [11,15]. This study showed that there were 45.7% of cholestatic infants in acute infection and 42.9% in past infection. In addition, the data showed that anti-HCMV IgM was in 45.7% and IgG was 88.6% of cholestatic infants. Other studies showed that IgM positive for HCMV in neonatal cholestasis in Sweden was 32.2% and IgG positive was 90% [22] and in Brazil, 28.9% was positive for IgM, both in intra and extra hepatic cholestasis [1]. Neonatal cholestasis in Egypt, IgM HCMV was positive in 12.4% [25]. In addition, other studies in India showed that anti-HCMV IgM was positive in 42% of patients and anti-HCMV IgG was positive in 84% of patients in neonatal cholestasis [24]. The IgM in primary infection of neonatal, showed the IgM reaches the peak at the first of 1 to 3 months, and later the titer begins to decrease [7], but persistent anti-HCMV IgM in the low level usually can be detected in more than 3 months or up to a year [7]. On the other hand, the maternal IgG of HCMV in infants will disappear at 8 months [2].

This study showed that there was concordance between anti-HCMV IgM with HCMV PCR from urine specimens with fair strength of agreement (0.246). It showed all infants with IgM positive were positive PCR from urine specimens. Furthermore, there was no concordance between anti-HCMV IgM with HCMV PCR from liver biopsy, that 12/16 (46.2%) infants with positive anti-HCMV IgM were positive for HCMV PCR. There were 4 infants who showed IgM positive and HCMV PCR negative. This may be because IgM can persist for 6 to 9 months after primary infection [11]. Therefore, IgM serology results are still positive while viral DNA is negative. In addition, there were 5 out of 19 infants with IgM negative, but PCR from liver and urine specimens were positive. This is in accordance with other studies which suggested that the serological examination of HCMV turned out to be a less accurate marker of HCMV infection in liver tissue [24]. The accuracy of serology for detecting HCMV antibodies was low [23]. The positivity of anti-HCMV IgM or HCMV DNA does not indicate the cause of cholestasis, but it implies that the virus may have influenced the severity of the original pathology [25].

This study showed the concordance of anti-HCMV IgG with HCMV PCR from liver and urine specimens with fair strength of agreement. Among 31 cholestatic infants with positive anti-HCMV IgG, there were 25 (96.2%) infants were positive for HCMV PCR from liver tissue specimens and 28 (93.3%) infants were positive for HCMV PCR from urine specimens. The liver and urine specimens of some infants showed HCMV PCR negative and IgG was positive. The presentation of IgG anti-CMV indicates a past infection, where anti-CMV IgG antibodies were produced for 2 weeks post-infection and persisted for years [11]. Data showed the infants were 3 to 5 months age. This suggested that the virus may have infected in the past. In addition, there were 3 of liver and 2 urine specimens with IgM, IgG, and HCMV DNA were negative. This might indicate that the infants were not infected with HCMV, while the cholestasis was caused by others etiologies [24].

In this study, HCMV PCR from urine specimen had sensitivity higher than specimen from liver, that was 92.31% with the accuracy diagnostic was 77%. HCMV PCR is a highly sensitive method for detecting HCMV in variable clinical samples [11]. In addition, urine specimens are easy to collect, non-invasive, and large amounts of viral shedding are found in body fluids including urine [11]. It is different from a liver biopsy. It is difficult, invasive, and painful, require the proper skills and radiological equipment. Therefore, urine sample was more feasible to use as specimen for PCR in diagnosing HCMV infection of cholestatic infants.

In this study, sensitivity and specificity of IgM anti-HCMV compared with HCMV PCR of liver biopsy specimens were 46.15% and 55.55%, respectively, with a diagnostic accuracy of 48.57%. In addition, anti-HCMV IgG still had a high sensitivity of 96.15% in the liver and 93.33 % in urine specimens. This is in accordance with previous studies which stated that the HCMV PCR test was more sensitive and specific than the anti-HCMV serological test [24]. Sensitivity and specificity of anti-HCMV IgM compared with HCMV PCR from liver samples were 69% and 61%, respectively [24]. The sensitivity and specificity of PCR is higher than that of antigenemia, the sensitivity can reach 100%, the specificity is 72-90%, the positive predictive value is 69-90%, and the negative predictive value is 100% [27,28]. In addition, these results indicates that serological examination, when compared with HCMV PCR from urine specimens, shows high diagnostic accuracy that more than 60%. Therefore, in remote areas or area that do not have PCR equipment, the serological examination can still be an option for detecting HCMV infection in cholestatic infants. However, the anti-HCMV serological examination cannot replace PCR [29], so in health centers that have an access to perform PCR, PCR remains a necessity in

diagnosing cholestatic infants because it has higher sensitivity and specificity.

## Conclusions

This study demonstrated a high prevalence of HCMV DNA in the urine and liver biopsy specimens of cholestatic infants. HCMV PCR in urine had higher sensitivity than in the liver with a diagnostic accuracy of about 77%. Considering this and the patient is an infant, urine is the more widely available specimen for use in the diagnosis of CMV infection in cholestatic infants.

This study found no concordance between IgM with the PCR liver, but there was concordance between IgM with the PCR urine, and between IgG with the PCR liver and urine. In addition, HCMV PCR test was more sensitive and specific than the anti-HCMV serological test which IgM compared with the PCR liver has sensitivity and specificity of about 50%, and compared with the urine PCR has a sensitivity of 53% and specificity of 100%, with the diagnostic accuracy of 60%. Furthermore, IgG compared with the PCR urine has a high sensitivity of 95% with a high accuracy diagnostic of more than 80%, but has a low specificity. Considering the vast territory of Indonesia which consists of thousands of islands, there are still many health centers that lack equipment to perform PCR. Therefore, serological examination is an option for diagnosing HCMV infection in infants with cholestasis. This can also be applied in other developing countries that have not yet reached PCR testing services.

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## Authors' Contributions

All of the authors contributed to reading and approved the final manuscript. Alphania Rahniayu: main idea, writing, and editing manuscript, histopathological diagnoses. Gondo Mastutik: main idea, laboratory examinations, writing, and editing manuscript, reviewing. Anny Setijo Rahaju: reviewing and histopathological diagnoses. Siti Eriaty Nur Ruslan: laboratory examinations. Priangga Adi Wiratama: statistical analysis. Erna Sulistiyani: reviewing manuscript. Bagus Setyoboedi: collecting patients.

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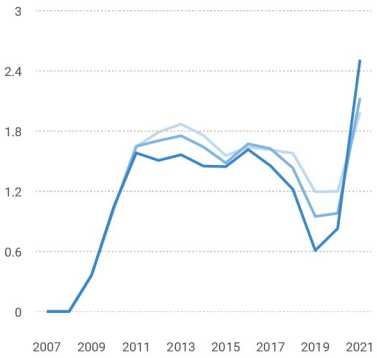


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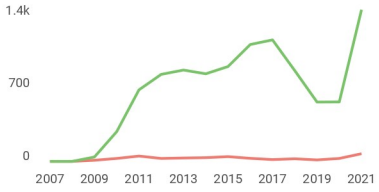


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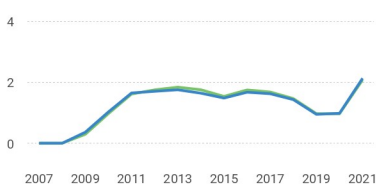


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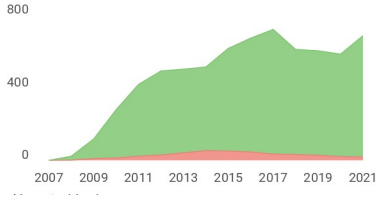


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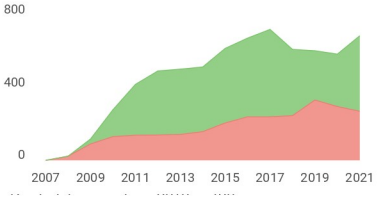



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