*Tropical Biomedicine* 43(2): 180-184 (2026)
https://doi.org/10.47665/tb.43.2.006


RESEARCH ARTICLE

# Diagnostic agreement between Simplexa direct real-time PCR and conventional nested PCR for detecting congenital cytomegalovirus in neonatal urine in Indonesia

**Pamungkas, J.[1], Wardhani, P.[2,6,7,8*], Husada, D.[3,6,7], Ernawati[4,6], Purnami, N.[5,6], Fitriah, M.[2,6]**

[1]Clinical Pathology Specialization Program, Faculty of Medicine, Universitas Airlangga, Surabaya, Indonesia
[2]Department of Clinical Pathology, Faculty of Medicine, Universitas Airlangga, Surabaya, Indonesia
[3]Department of Pediatrics, Faculty of Medicine, Universitas Airlangga, Surabaya, Indonesia
[4]Department of Obstetrics and Gynecology, Faculty of Medicine, Universitas Airlangga, Surabaya, Indonesia
[5]Department of Ear, Nose, and Throat, Faculty of Medicine, Universitas Airlangga, Surabaya
[6]Dr. Soetomo General Academic Hospital, Surabaya, Indonesia
[7]Institute of Tropical Diseases, Universitas Airlangga, Surabaya, Indonesia
[8]Postgraduate School, Universitas Airlangga, Surabaya, Indonesia
*Corresponding author: puspa-w-2@fk.unair.ac.id

**ARTICLE HISTORY**

Received: 18 September 2025
Revised: 3 April 2026
Accepted: 3 April 2026
Published: 30 June 2026

**ABSTRACT**

Congenital cytomegalovirus (cCMV) infection is a major cause of neurodevelopmental impairment and sensorineural hearing loss worldwide, particularly in low- and middle-income countries where standardized molecular screening programs are not routinely implemented. Early laboratory confirmation within the first 21 days of life is essential to differentiate congenital from postnatal infection. This study evaluated the diagnostic agreement between the Simplexa cCMV Direct real-time PCR assay and conventional nested PCR for detecting CMV DNA in neonatal urine specimens in Indonesia. A cross-sectional diagnostic agreement study was conducted between September and December 2023 at a tertiary referral hospital. Urine samples from 87 neonates aged ≤ 21 days with clinical suspicion of congenital infection were tested using both assays. The Simplexa assay detected 29 positive and 58 negative samples, while conventional nested PCR identified 28 positive and 59 negative samples. Agreement was assessed using Cohen's kappa coefficient, McNemar's test, and positive and negative agreement rates. The assays demonstrated near-perfect agreement ($\kappa = 0.87$; 95% CI: 0.75–0.98; $p < 0.001$). Positive and negative agreement were 91.2% and 95.7%, respectively, with no statistically significant difference between methods (McNemar $p = 1.00$). Simplexa Direct real-time PCR showed high agreement with conventional nested PCR while offering operational advantages including simplified workflow and reduced contamination risk. These findings support the implementation of automated direct molecular platforms for neonatal cCMV diagnosis in tropical and resource-limited healthcare settings.

**Keywords**: congenital cytomegalovirus; neonatal infection; real-time PCR; conventional nested PCR; infectious disease.

## INTRODUCTION

Cytomegalovirus (CMV), a member of the Herpesviridae family, is the leading cause of congenital viral infection worldwide and a major non-genetic cause of sensorineural hearing loss and neurodevelopmental impairment in children (Pinninti & Boppana, 2022; Pesch & Schleiss, 2022; Salomè *et al*., 2023). The estimated birth prevalence of congenital CMV (cCMV) ranges from 0.5–0.7% in high-income countries and may exceed 1–2% in low- and middle-income countries (LMICs), where maternal seroprevalence is high (Ssentongo *et al*., 2021; Salomυ *et al*., 2023). Although most infected neonates are asymptomatic at birth, approximately 15–20% develop permanent sequelae, highlighting the importance of early detection.

Because clinical manifestations are nonspecific and overlap with other congenital infections, laboratory confirmation is essential. Serological testing in neonates is unreliable due to maternally transferred IgG antibodies and variable IgM responses (Pinninti & Boppana, 2022; Leber, 2024).

Nucleic acid amplification testing (NAAT), particularly polymerase chain reaction (PCR) of urine or saliva collected within the first 21 days of life, is therefore considered the diagnostic standard for distinguishing congenital from postnatal infection (Leber, 2024; Pinninti & Boppana, 2022). According to the Centers for Disease Control and Prevention (CDC), laboratory confirmation of congenital CMV infection should be performed within the first

21 days of life to differentiate congenital from postnatal infection (CDC, 2024).

Multiple molecular platforms are available for cCMV detection, differing in workflow complexity, contamination risk, and scalability. Conventional nested PCR provides high analytical sensitivity but requires nucleic acid extraction and open-tube processing, increasing contamination risk and procedural variability (Leber, 2024). In contrast, automated direct real-time PCR systems, such as the Simplexa Congenital CMV Direct assay, enable extraction-free amplification within a closed platform, reducing hands-on time and improving standardization. Previous studies have demonstrated high concordance between this assay and established molecular methods (Fernholz *et al*., 2023).

In Indonesia and other LMICs, standardized automated molecular diagnostics for congenital CMV are not widely implemented, and many laboratories continue to rely on conventional or in-house PCR assays (Salomè *et al*., 2023; Leber, 2024). In the absence of universal newborn screening, targeted molecular testing in high-risk neonates remains the most practical diagnostic approach (Pesch & Schleiss, 2022; Salomè *et al*., 2023). Locally generated validation data are therefore needed to support evidence-based laboratory implementation.

This study aimed to evaluate the diagnostic agreement between the Simplexa cCMV Direct real-time PCR assay and conventional nested PCR for detecting CMV DNA in neonatal urine specimens in a tertiary referral hospital in Indonesia.

## MATERIALS AND METHODS

This cross-sectional diagnostic agreement study was conducted between September and December 2023 at Dr. Soetomo General Academic Hospital, Surabaya, Indonesia. During the study period, 92 consecutive urine specimens were collected from neonates undergoing evaluation for suspected congenital infection. The study workflow, including patient recruitment and diagnostic sample selection, is presented in Figure 1.

Of these, 87 samples met the eligibility criteria and were included in the analysis. Inclusion criteria were age ≤21 days at the time of urine collection, minimum urine volume of 2 mL, valid internal control amplification in both assays, and the presence of at least one clinical feature suggestive of congenital infection, including prematurity, intrauterine growth restriction, neonatal jaundice, hepatosplenomegaly, thrombocytopenia, or abnormal neuroimaging findings. Samples were excluded if the neonate was older than 21 days, the specimen volume was insufficient, internal control amplification was invalid, or postnatal CMV infection was suspected.

Clinical and demographic data were obtained from medical records and the hospital Laboratory Information System. The study protocol was approved by the Ethics Committee of Dr. Soetomo General Academic Hospital (No. 0694/KEPK/VI/2023), and written informed consent was obtained from parents or legal guardians.

Detection of CMV DNA using the Simplexa cCMV Direct real-time PCR assay (DiaSorin Molecular, Italy) was performed on the LIAISON® MDX platform according to the manufacturer's instructions. Briefly, 50 µL of unprocessed urine was added to the sample well of a Direct Amplification Disc, followed by 50 µL of reaction mix. Amplification was carried out for 45 cycles with real-time fluorescence detection. An internal control was included in each reaction. Results were interpreted automatically as "Detected", "Not Detected", or "Invalid". Cycle threshold (Ct) values were recorded for descriptive purposes.

Conventional nested PCR was performed following DNA extraction using the QIAamp DNA Mini Kit (QIAGEN, Germany). Amplification was performed in two rounds using the MIE4/MIE5 and IE1/IE2 primer pairs. Primer sequences were as follows: MIE4 (5'-CCAAGCGGCCTCTGATAACCAAGCC-3'), MIE5 (5'-CAGCACC ATCCTCCTCTTCCTCTGG-3'), IE1 (5'-CCCCCTCTGCTCCACGTAGCC-3'), and IE2 (5' -CAGCGTGTCCGGGTTGAGACC-3'). Expected amplicon sizes were 435 bp for the first-round PCR and 161 bp for the nested PCR product. Thermal cycling consisted of initial denaturation at 94°C for 5 minutes, followed by 35 cycles of denaturation, annealing at 57°C, and extension, with a final extension step at 72°C for 5 minutes. Amplification products were analysed by agarose gel electrophoresis. A schematic comparison of the workflows for conventional nested PCR and Simplexa real-time PCR is shown in Figure 2.

Diagnostic agreement between the two methods was evaluated using Cohen's kappa coefficient with 95% confidence intervals. Paired differences were assessed using McNemar's test. Positive and negative agreement were calculated based on concordant and discordant results. Statistical analysis was performed using SPSS version 26.0 (IBM Corp., Armonk, NY, USA), with $p < 0.05$ considered statistically significant.

## RESULTS

### Patient characteristics

A total of 87 neonatal urine samples were analyzed. The mean age of the neonates was 8.9 ± 6.3 days. Of these, 52 (59.8%) were male and 35 (40.2%) were female. Most samples (47/87, 54.0%) were collected within the first 7 days of life, followed by 24 (27.6%) collected between days 8–14 and 16 (18.4%) between days 15–21 (Table 1). Common clinical features included prematurity, low birth weight, and neonatal jaundice. Detection rates did not significantly differ across age groups or sex ($p > 0.05$).

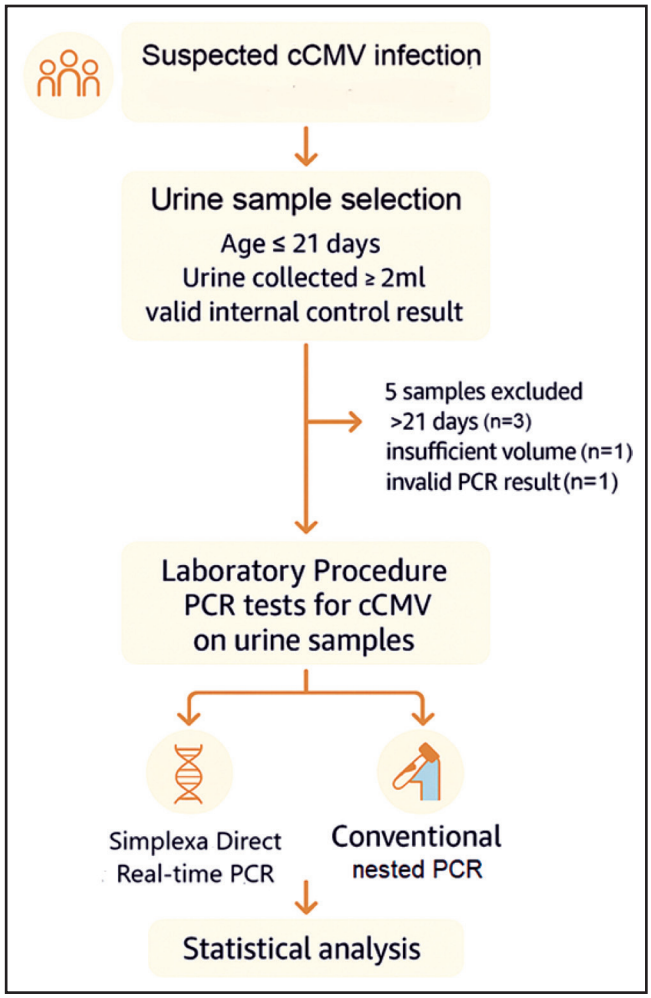


**Figure 1.** Flowchart of patient recruitment and diagnostic sample selection.

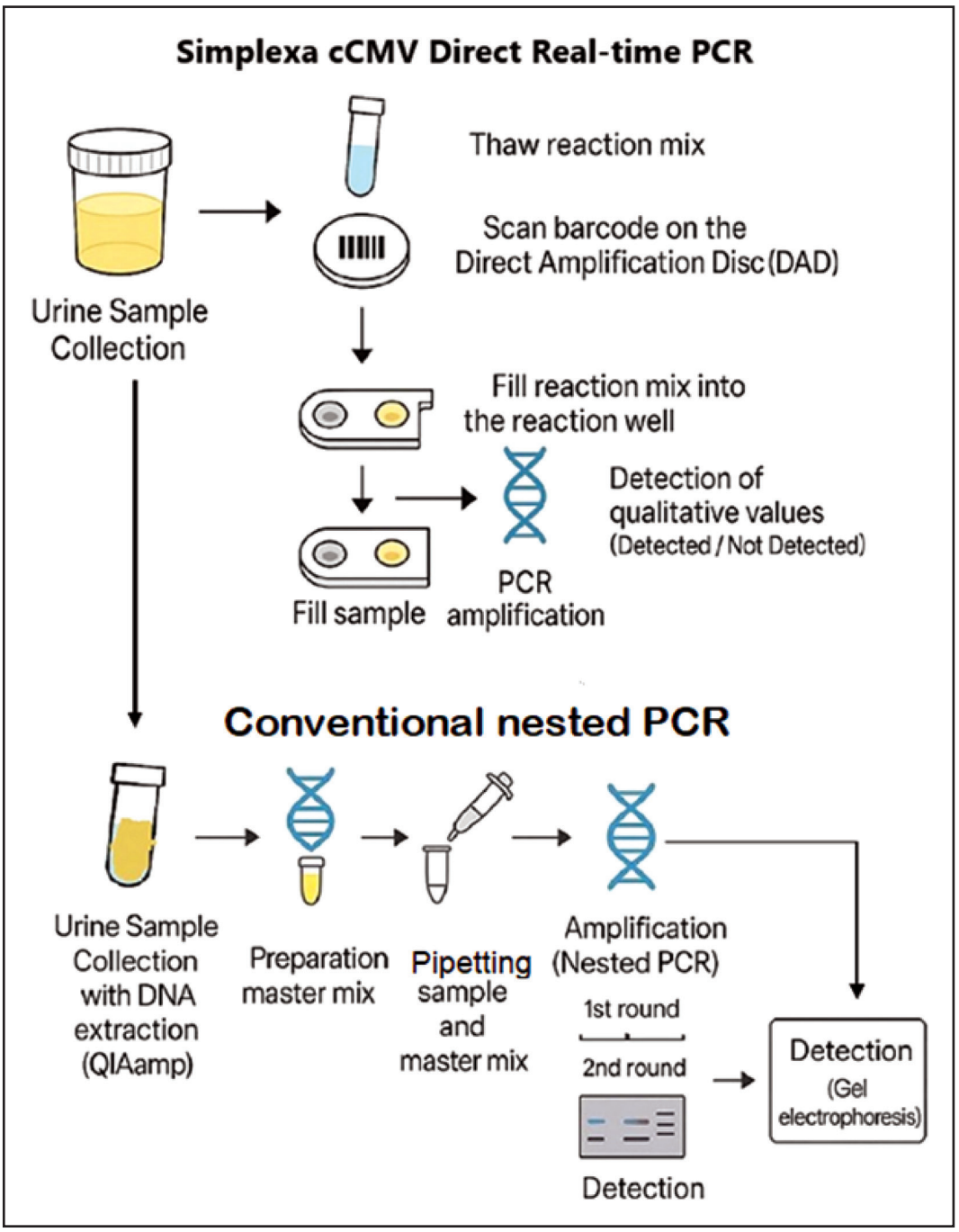


**Figure 2.** Workflow comparison between conventional nested PCR and Simplexa Real-time PCR.

**Table 1.** Age and sex distribution according to conventional nested PCR and Simplexa real-time PCR results

| | | Conventional nested PCR (n=87)(%) | | Simplexa cCMV Direct Real-time PCR (n=87)(%) | |
|---|---|---|---|---|---|
| | | Positive | Negative | Positive | Negative |
| Age (Days), | mean±SD 8.90±6.29 | | | | |
| | 0 – 7 | 16 (18.4) | 31 (35.6) | 16 (18.4) | 31 (35.6) |
| | 8 – 14 | 8 (9.2) | 16 (18.4) | 8 (9.2) | 16 (18.4) |
| | 15 – 21 | 4 (4.6) | 12 (13.8) | 5 (5.7) | 11 (12.6) |
| Sex | Male | 17 (19.5) | 35 (40.2) | 17 (19.5) | 35 (40.2) |
| | Female | 11 (12.6) | 24 (27.6) | 12 (13.8) | 23 (26.4) |

NB: Percentages are calculated based on total sample size (n=87).

**Diagnostic results of Simplexa real-time PCR and conventional nested PCR**

The Simplexa cCMV Direct real-time PCR assay detected 29 positive samples (33.3%) and 58 negative samples (66.7%); conventional nested PCR identified 28 positive (32.2%) and 59 negative (67.8%) samples. Among the 87 paired results, 26 samples were positive by both methods, and 56 were negative by both. Five discordant results were observed: three samples were Simplexa-positive/conventional-negative and two were Simplexa-negative/conventional-positive (Table 2).

**Diagnostic agreement analysis**

The diagnostic agreement analysis between Simplexa real-time PCR and conventional nested PCR is summarized in Figure 3. Inter-method agreement was high. Cohen's kappa coefficient was 0.87

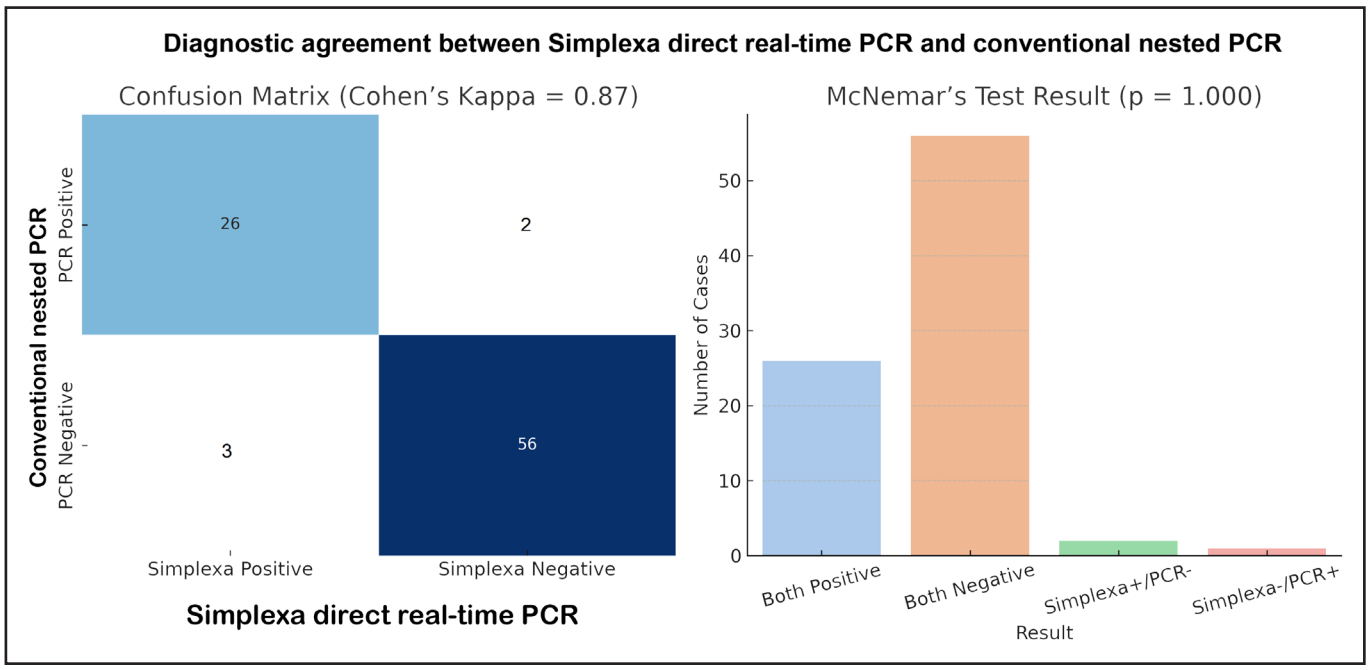


**Figure 3.** Diagnostic agreement between Simplexa Real-time PCR and conventional nested PCR (Cohen's kappa, McNemar test, agreement rate).

**Table 2.** Agreement between Simplexa Real-time PCR and Conventional nested PCR using Cohen's Kappa statistic

| | | Real-time PCR | | *P Value* | *Kappa Value* | Interpretation |
|---|---|---|---|---|---|---|
| | | Negative | Positive | | | |
| Conventional nested PCR | Negative | 56 | 3 | < 0.001 | 0.870 | Near-perfect agreement |
| | Positive | 2 | 26 | | | |

**Table 3.** McNemar's test for paired comparison between Simplexa Real-time PCR and Conventional nested PCR

| | | Real-time PCR | | *P Value* | Interpretation |
|---|---|---|---|---|---|
| | | Negative | Positive | | |
| Conventional nested PCR | Negative | 56 | 3 | 1.00 | No Significant Difference |
| | Positive | 2 | 26 | | |

**Table 4.** Positive and Negative Agreement Between Simplexa Real-time PCR and Conventional nested PCR

| Agreement Type | Formula | Value | Confidence Interval (95%) |
|---|---|---|---|
| Positive Agreement | 2a / (2a+b+c) | 91.2% | 81.1% – 96.2% |
| Negative Agreement | 2d / (2d+b+c) | 95.7% | 90.4% – 98.2% |

Nb: a = 26 (concordant positive); b = 3; c = 2; d = 56 (concordant negative). Discordant pairs were defined as b = Simplexa-positive/conventional-negative and c = Simplexa-negative/conventional-positive.

(95% CI: 0.75–0.98; p < 0.001) (Table 2). McNemar's test showed no statistically significant difference between the two assays (p = 1.00) (Table 3). Positive agreement was 91.2% (95% CI: 81.1%–96.2%), and negative agreement was 95.7% (95% CI: 90.4%–98.2%) (Table 4).

**Cycle threshold (Ct) values in real-time PCR**

Among the 29 Simplexa-positive specimens, Ct values ranged from 24.5 to 31.4. Higher Ct values were observed in discordant samples.

**Invalid results**

One specimen was excluded due to an invalid Simplexa result, characterized by the absence of amplification in both the CMV target and internal control.

## DISCUSSION

The present study demonstrates high diagnostic agreement between the Simplexa cCMV Direct real-time PCR assay and conventional nested PCR for detecting CMV DNA in neonatal urine specimens. The near-perfect kappa coefficient and non-significant McNemar test indicate strong inter-method reliability without evidence of systematic bias. These findings provide locally generated validation data supporting the feasibility of automated extraction-free molecular platforms in routine clinical laboratory practice within a middle-income country setting.

The CMV detection proportion in this cohort ranged from 32.2% to 33.3%, depending on the assay used, with CMV identified in 29/87 samples (33.3%) by Simplexa real-time PCR and in 28/87 samples (32.2%) by nested PCR, which is substantially higher than the estimated global prevalence of congenital CMV infection in the general population (0.5–0.7% of live births)(Ssentongo *et al*., 2021; Salomè *et al*., 2023). This difference reflects the selectively enriched study population, consisting of neonates undergoing targeted diagnostic evaluation for suspected congenital infection in a tertiary referral hospital. Therefore, the observed detection rate represents a high-risk clinical subgroup rather than population prevalence (Pesch & Schleiss, 2022).

The discordant results observed in this study likely reflect methodological differences between extraction-based nested PCR and direct real-time amplification platforms. While nested PCR offers high analytical sensitivity, its reliance on nucleic acid extraction and open-tube processing may introduce variability and increase contamination risk (Leber, 2024). In contrast, extraction-free real-time PCR operates within a closed system, reducing manual handling and improving workflow standardization (Fernholz *et al*., 2023). The balanced distribution of discordant pairs and the absence of statistically significant disagreement suggest that these discrepancies represent random analytical variation near the detection threshold rather than systematic bias.

Higher Ct values observed in discordant samples further support the possibility of low viral burden near the analytical limit of detection. However, as quantitative viral load analysis was not performed, these findings should be interpreted cautiously.

Beyond analytical performance, operational considerations are important. Automated direct real-time PCR platforms offer simplified workflows, shorter turnaround times, and reduced contamination risk. These features may be particularly beneficial in high-volume tertiary care settings, where timely diagnosis within the first 21 days of life is critical for distinguishing congenital from postnatal infection (CDC, 2024; Leber, 2024). The low invalid rate observed in this study also suggests acceptable analytical robustness in routine practice.

In many low- and middle-income countries, including Indonesia, universal newborn screening for congenital CMV has not been widely implemented (Pesch & Schleiss, 2022; Salomè *et al*., 2023). Targeted molecular testing in clinically suspected neonates therefore remains a pragmatic diagnostic approach. In this context, locally validated and operationally feasible diagnostic platforms are essential to support evidence-based laboratory implementation.

Several limitations should be acknowledged. This was a single-center study with a relatively modest sample size. In addition, no independent reference standard was available; therefore, sensitivity and specificity could not be determined. Quantitative viral load analysis was also not performed, limiting further evaluation of discordant results.

Future multicenter studies incorporating larger cohorts, quantitative viral load assessment, and clinical outcome correlation are warranted to further define the role of automated molecular platforms in congenital CMV diagnosis. Nevertheless, the present findings support the analytical reliability and practical feasibility of direct real-time PCR for use in high-risk neonatal populations.

## CONCLUSION

This study demonstrates that an automated direct real-time PCR platform provides diagnostic agreement comparable to that of conventional nested PCR for detecting congenital CMV in neonatal urine specimens. The observed agreement supports the analytical reliability of direct amplification methods in routine neonatal diagnostics.

The simplified workflow and reduced contamination risk associated with closed-system automation may improve laboratory efficiency and standardization, particularly in resource-limited or high-demand settings. These findings support the integration of automated molecular platforms into targeted diagnostic strategies for congenital CMV in similar healthcare contexts.

## ACKNOWLEDGEMENTS

The authors thank the clinical and laboratory staff of Dr. Soetomo General Academic Hospital for their valuable assistance in sample collection and technical support. This research was funded by the Ministry of Education, Culture, Research, and Technology of the Republic of Indonesia under the 2023 research grant scheme (Register No. 0536/E5/PG.02.00/2023).

**Conflict of Interests**

The authors declare that they have no conflict of interests.

## REFERENCES

Centers for Disease Control and Prevention (CDC). (2024). Congenital Cytomegalovirus (cCMV) Infection and Disease 2024 Case Definition. Available at: https://ndc.services.cdc.gov/case-definitions/congenital-cytomegalovirus-ccmv-infection-and-disease-2024/. Accessed 20 May 2026.

Fernholz, E.C., Vidal-Folch, N. & Hasadsri, L. (2023). Rapid and direct detection of congenital cytomegalovirus using a commercial real-time PCR assay. *Journal of Clinical Microbiology* **61**: e01781-22. https://doi.org/10.1128/JCM.01781-22

Leber, A.L. (2024). Maternal and congenital human cytomegalovirus infection: laboratory testing for detection and diagnosis. *Journal of Clinical Microbiology* **62**: e00313-23. https://doi.org/10.1128/JCM.00313-23

Pesch, M.H. & Schleiss, M.R. (2022). Emerging concepts in congenital cytomegalovirus. *Pediatrics* **150**: e2021055896. https://doi.org/10.1542/peds.2021-055896

Pinninti, S.G. & Boppana, S.B. (2022). Congenital cytomegalovirus infection diagnostics and management. *Current Opinion in Infectious Diseases* **35**: 436-441. https://doi.org/10.1097/QCO.0000000000000874

Salomè, S., Corrado, F.R., Mazzarelli, L.L., Maruotti, G.M., Capasso, L., Blázquez-Gamero, D. & Raimondi, F. (2023). Congenital cytomegalovirus infection: the state of the art and future perspectives. *Frontiers in Pediatrics* **11**: 1276912. https://doi.org/10.3389/fped.2023.1276912

Ssentongo, P., Hehnly, C., Birungi, P., Roach, A., Spady, J., Fronterre, C., Wang, M., Murray-Kolb, L.E., Al-Shaar, L., Chinchilli, V.M. *et al*. (2021). Congenital cytomegalovirus infection burden and epidemiologic risk factors in countries with universal screening: a systematic review and meta-analysis. *JAMA Network Open* **4**: e2120736. https://doi.org/10.1001/jamanetworkopen.2021.20736