DOI: 10.7860/JCDR/2025/80393.21933

Original Article

Paediatrics Section

Clinical Screening of Cytomegalovirus Infection beyond 21 Days in the Absence of its Routine Screening in Resource Limited Settings: A Cross-sectional Study

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ABSTRACT

Introduction: Testing for congenital Cytomegalovirus (cCMV) infection is not part of routine care in resource-limited settings. Consequently, the diagnosis is often missed because most infections are asymptomatic and when present, symptoms are non specific. Diagnosis is required to prevent long-term sequelae.

Aim: To introduce a clinical screening strategy for detecting possible CMV infections, including both congenital and postnatal infections among symptomatic neonates/infants presenting after 21 days.

Materials and Methods: In this cross-sectional study, 387 subjects with clinical signs or symptoms compatible with congenital or postnatal CMV infection from birth to 365 days of life were included from three metropolitan hospitals in Kolkata, West Bengal, India, from January 2018 to March 2020. CMV infection was determined by Polymerase Chain Reaction (PCR) analysis of the urine. The Constellation of Symptoms (COS) was employed as a clinical screening tool for subjects older than 21 days. The Chi-square test was used to compare categorical variables, while the Mann-Whitney U test assessed differences in numerical data.

Results: Of the 387 subjects with compatible signs or symptoms, 126 (32.6%) tested positive for CMV. Among the CMV-infected group, 8 (6.3%) presented within 21 days, while 118 (93.7%) presented after 21 days. CMV-infected infants had slightly lower birth weight and younger age at presentation compared with CMV-negative infants, though the differences were not statistically significant. The presence of individual symptoms of CMV infection did not differ significantly between CMV-positive and CMV-negative groups (p-value>0.05). However, the COS was significantly associated with CMV positivity (p-value<0.001). The sensitivity and specificity of COS were in the ranges of 77%-81% and 40%-44%, respectively. The Positive Predictive Value (PPV) and Negative Predictive Value (NPV) were in the ranges of 38%-44% and 75%-82%, respectively.

Conclusion: The COS-based strategy will be a valuable tool for screening symptomatic CMV infections in individuals up to one year of age in resource-limited settings where routine CMV screening is unavailable.

Keywords: Constellation of symptoms, Cytomegalovirus, Late diagnosis, Polymerase chain reaction

INTRODUCTION

The congenital CMV infection (cCMV) is the most prevalent foetal viral infection, with an estimated global birth prevalence of 0.7% [1]. Its prevalence in developing countries remains high, ranging from 0.6-6.1% and maternal CMV seroprevalence ranges from 84-100% [2]. A recent review showed that the infection burden is threefold greater in low- and middle-income countries than in high-income countries [3]. Approximately 10-15% of cCMV infections are symptomatic, while the remainder are asymptomatic at birth [4]. Although any infant with cCMV infection is potentially at risk for sequelae, symptomatic infants have the highest risk of developing neurodevelopmental sequelae [1,5]. Thus, in settings where routine screening is not practiced, diagnosis relies on healthcare providers considering and performing appropriate testing in infants with compatible clinical manifestations. Clinical characteristics of symptomatic cCMV include cholestatic jaundice, hepatosplenomegaly, thrombocytopenia, microcephaly, chorioretinitis and Central Nervous System (CNS) anomalies [6]. The disease manifestations vary from mild and transient to severe and life-threatening. Term infants with perinatally or postnatally acquired CMV infection are almost always asymptomatic. In contrast, premature infants are at risk of developing fever, pneumonitis, thrombocytopenia, lymphocytosis, encephalitis and hepatosplenomegaly [7].

The diagnosis of cCMV infection relies on tests performed within the first 21 days of life [8]. Current international guidelines recommend targeted PCR screening of neonates within the first 21 days of life who are at the highest risk of sequelae (e.g., maternal serology showing a primary infection in the first trimester or symptomatic neonates) [9,10]. This targeted screening aims to identify neonates at the highest risk for congenital infection early, allowing timely diagnosis and management to reduce long-term sequelae. Screening strategies should balance country-specific cost-effectiveness with the risk of foetal infection. In resource-limited countries such as India, maternal CMV seroprevalence is almost 100%, reflecting widespread exposure among pregnant women [11,12]. Still, routine screening for CMV infection among infants is not practiced. Symptomatic CMV-infected infants, such as those with Sensorineural Hearing Loss (SNHL), are most commonly seen at medical facilities after 21 days of life [13]. Clinical screening criteria are needed to ensure the appropriate and cost-effective use of confirmatory PCR testing.

No published studies have specifically examined possible congenital and postnatal CMV infections in infants presenting after 21 days of life, especially in resource-limited settings. Most research and screening efforts focus on diagnosing cCMV within the first 21 days and ignore symptom-based screening for late-presenting infants, hindering early diagnosis and care [14-16]. The current study is among the first to use a symptom-based approach to identify CMV infection in infants beyond the neonatal period, addressing a major gap where newborn screening is unavailable. Hence, the present

study was conducted to introduce a clinical screening strategy for detecting possible CMV infections, which includes both congenital and postnatal infections among symptomatic neonates/infants presenting after 21 days.

MATERIALS AND METHODS

An observational cross-sectional study was performed at the Indian Council of Medical Research-National Institute of Cholera and Enteric Diseases, Virus Laboratory Division, Kolkata, India. Subjects were enrolled from three metropolitan hospitals: Dr. B. C. Roy Postgraduate Institute of Paediatric Sciences, Kolkata; Calcutta Medical College and Hospital, Kolkata; and Nil Ratan Sircar Medical College and Hospital, Kolkata, over two years and three months, from January 2018 to March 2020. The Institutional Ethics Committee and the three hospital ethics committees approved the study (Institute IEC approval number A-1/2018-IEC; BCH/ME/PR/3499; MC/KOL/IEC/NON-SPON/415/06/19; and NMC/2618, respectively) in accordance with the 1964 Helsinki Declaration. Informed consent was obtained from the parents.

Inclusion criteria: All cases with signs and symptoms suggestive of cCMV infection were included. Infants from birth to 365 days of age were tested with CMV PCR if they had any of the following symptoms: cholestatic jaundice, hepatosplenomegaly, microcephaly, chorioretinitis, neurodevelopmental delay, or abnormalities on neuroimaging.

Exclusion criteria: Subjects with bacterial infections and TORCH infections other than CMV, such as Toxoplasma, Rubella, Herpes Simplex, viral hepatitis, and Human Immunodeficiency Virus-1 (HIV-1) infections, were excluded from the study.

Sample size: A formal sample size calculation was not performed due to the lack of directly comparable studies focusing solely on symptomatic CMV infection in neonates/infants beyond 21 days. Most available literature includes both symptomatic and asymptomatic cohorts that do not apply to our narrowly defined target population. Given these constraints, a pragmatic approach was adopted. All eligible symptomatic infants presenting to the study sites during the predefined study period were recruited by convenience sampling.

Data collection: All CMV-suspected subjects were evaluated based on medical history and comprehensive medical, neurological and developmental examinations by physicians to confirm the diagnosis. All investigations, including complete blood count; liver function tests; TORCH panel testing; and routine blood cultures, were performed. Ophthalmoscopy was used to detect chorioretinitis, and auditory brainstem response testing was used to detect SNHL. Cranial ultrasonography, computed tomography, and/or magnetic resonance imaging were performed when CNS involvement was suspected [17].

Clinical screening strategy and disease severity classification: To detect cCMV infection, PCR testing of urine was performed within the first 21 days. Beyond 21 days, a PCR-positive result is referred to as postnatal CMV infection. To identify cases of possible CMV infection, including congenital and postnatal infection beyond 21 days, a symptom-guided clinical screening approach was employed. The screening strategy consists of a COS, including characteristic CMV-related symptoms, namely cholestatic jaundice, hepatosplenomegaly, microcephaly, and abnormalities on neuroimaging, mainly CNS calcifications, to detect possible CMV infection. The COS was considered present if the subject presented with either a single symptom or multiple characteristic symptoms. Disease severity was classified into mild and moderate-to-severe symptomatic cases according to the consensus guidelines [18]. Briefly, mild disease is characterised by isolated findings such as mild hepatosplenomegaly, transient thrombocytopenia, and the absence of significant neurological involvement. In contrast, moderate-to-severe disease involves more extensive, often systemic manifestations, including SNHL, microcephaly, chorioretinitis, or CNS abnormalities.

Sample collection and Deoxyribonucleic Acid (DNA) isolation: A urine sample was used for confirmatory diagnosis of CMV infection [19]. Approximately 2-3 mL of urine was collected from CMV-suspected subjects in a sterile container. The urine was centrifuged at 4000 × g for 15 minutes; the supernatant was discarded, and the pellet was processed for DNA extraction using the DNASure Tissue Mini Kit (NP-61307, Genetix) following the manufacturer's instructions.

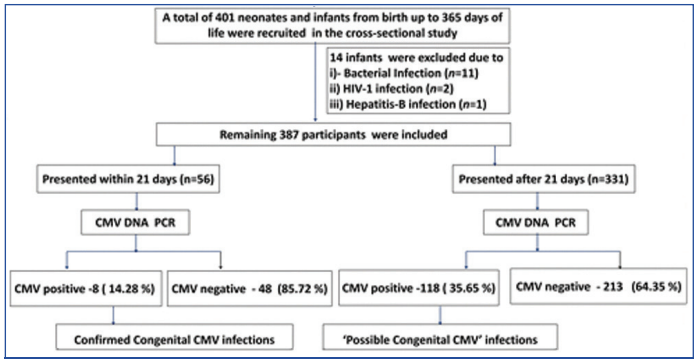
Qualitative PCR: CMV infection in infants up to 365 days was confirmed by qualitative PCR on the isolated DNA via amplification of the UL83 gene (216 bp product) [20]. To amplify the gene, a 25 µL reaction mixture containing 12.5 µL of 2× master mix (Takara, Japan), 3 µL of isolated DNA, 1 µL each of forward and reverse primers (10 µM), and 7.5 µL of PCR-grade water was used. The thermal cycling conditions (GeneAmp PCR system 9700; Applied Biosystems, USA) were: 94°C for 5 minutes; 35 cycles of 94°C for 30 seconds, 55°C for 45 seconds, and 72°C for 30 seconds; and final extension at 72°C for 5 minutes. The AD169 CMV strain was used as the positive control. The primer sequences were manually designed in silico using Primer-3 software in the laboratory and obtained from Eurofins Genomics Pvt., Ltd., India: Forward primer 5′-GATCTTGCCCGGTTTGATTA-3′ and Reverse primer 5′-ATGCAGGTGATAGGTGACCA-3′.

STATISTICAL ANALYSIS

Categorical variables were presented as frequencies and percentages. To compare two categorical variables, the Chi-square test was used. The normality of data was assessed with the Shapiro-Wilk test. The Mann-Whitney U test was used to assess differences between two non parametric independent groups. The validity of the COS was calculated using sensitivity, specificity, Positive Predictive Value (PPV) , Negative Predictive Value (NPV) and positive and negative likelihood ratios using MedCalc software [21]. A p-value <0.05 was considered to indicate statistical significance. The statistical analysis was performed using Statistical Package for the Social Sciences (SPSS) version 21.0.

RESULTS

A total of 401 participants were recruited based on suggestive clinical features. Fourteen cases (11 with bacterial infection, 2 with HIV-1 infection, and 1 with hepatitis B infection) were excluded, and the remaining 387 patients were included in the study [Table/Fig-1]. Among the study subjects (n=387), 126 (32.6%) were CMV-positive, and 261 (67.4%) were CMV-negative. Fifty-six of 387 participants (14.5%) presented within 21 days of life; 8 (14.3%) of whom tested positive for CMV and were therefore confirmed to have cCMV. Of the 331 children who presented after 21 days of life, 118 (35.6%) tested positive for CMV. The demographic profile showed that CMV-infected infants had a slightly lower mean birth weight (2380±532 g vs. 2460±541 g, p-value=0.130) and were younger at presentation (97.1±69.5 days vs. 101.1±93.5 days,



[Table/Fig-1]: Flow diagram of study participants showing CMV DNA PCR outcomes and classification of congenital vs. possible CMV infection.

p-value=0.117) [Table/Fig-2]. There was no significant difference in individual symptoms or signs between infants with or without CMV infection [Table/Fig-3].

Total included participants (n=387)			
	CMV Positive, n=126	CMV Negative, n=261	p-value
Presented within 21 days (n=56)	8 (14.28)	48 (85.72)	0.002
Presented after 21 days (n=331)	118 (35.65)	213 (64.35)	
Neonate/Infant demographics			
Gender			
Male	77 (61.10)	157 (60.20)	0.857
Female	49 (38.9)	104 (39.8)	
Age (days)	97.056±69.519	101.149±93.529	0.117
Birth weight (grams)	2380.373±532.241	2460.031±541.449	0.130
Gestation age			
Preterm (<37 weeks)	34 (26.98)	73 (27.96)	0.979
Term (37-42 weeks)	88 (69.84)	180 (68.96)	
Post term (>42 weeks)	4 (3.17)	8 (3.06)	
Maternal demographics			
Age (years)	24.032±4.562	23.314±4.330	0.150
Delivery mode			
Normal vaginal delivery	53 (42.06)	119 (45.59)	0.513
Lower segment caesarean section	73 (57.94)	142 (54.41)	
Gravida			
Primigravida	67 (53.17)	138 (52.87)	0.956
Multigravida	59 (46.83)	123 (47.13)	
Para			
Primipara	67 (53.17)	137 (52.49)	0.899
Multipara	59 (46.83)	124 (47.51)	

[Table/Fig-2]: Baseline demographics of study participants.
Categorical data are presented as n (%), with comparisons made using the Chi-square test. Numerical data are presented as mean±SD and compared using the Mann-Whitney U test (non-parametric); p<0.05 indicates a significant difference

Thus, a COS was used to screen for possible CMV infection. The presence of the COS significantly differed between the CMV-positive and CMV-negative groups in the age range from birth to 365 days (p-value<0.001) and from 22 to 365 days (p-value<0.001). Therefore, this constellation can identify both congenital CMV (cCMV) and possible CMV infections, including congenital and postnatal infections up to 365 days of age. The infants aged 22 to 365 days were further stratified into two groups: early (22-90 days) and late infancy (91-365 days) to assess whether the proposed constellation retained diagnostic relevance within 365 days when CMV-related clinical signs may appear. The presence of COS remained significant from 22 to 90 days (p-value=0.006) and from 91 to 365 days (p-value=0.008) [Table/Fig-4].

The discriminative properties of the COS, i.e., sensitivity and specificity, were 77-81% and 40-44%, respectively. The sensitivity was reasonable, but the specificity was low. The predictive abilities, such as the PPV and NPV, were 38%-44% and 75%-82%, respectively. The PPV was low, but the NPV was reasonable. Diagnostic accuracy measures indicate that the use of this constellation as a screening test seems feasible [Table/Fig-4].

The guidelines were applied to determine whether there was any relationship between the disease severity of CMV infection and the presence of COS. Analysis was performed to determine these relationships [Table/Fig-5]. No significant relationship was found between disease severity and COS.

S. No.	Symptoms	CMV Positive, n=126	CMV negative, n=261	p-value
Symptoms related to the Central Nervous System (CNS symptoms)				
1	Chorioretinitis	4 (3.2)	1 (0.4)	0.207
2	Sensorineural Hearing Loss (SNHL)	4 (3.2)	3 (1.1)	0.724
3	Hydrocephalus	2 (1.6)	1 (0.4)	0.618
4	Microcephaly	15 (11.9)	21 (8.0)	0.497
5	Seizure/Convulsion	11 (8.7)	32 (12.3)	0.54
6	Cataract	2 (1.6)	2 (0.8)	0.681
7	Ventriculomegaly	0	1 (0.4)	0.626
8	CNS Calcification	5 (4.0)	8 (3.1)	0.724
9	Neurodevelopmental delay	4 (3.2)	9 (3.4)	0.889
Symptoms not related to Central Nervous System (non CNS symptoms)				
1	Neonatal cholestasis	53 (42.1)	82 (31.4)	0.429
2	Hepatosplenomegaly	52 (41.3)	85 (32.6)	0.511
3	Neonatal hepatitis	7 (5.6)	27 (10.3)	0.436
4	Pneumonia	17 (13.5)	49 (18.8)	0.536
5	Respiratory distress syndrome	13 (10.3)	38 (14.6)	0.545
6	Thrombocytopenia	10 (7.9)	28 (22.2)	0.647
7	Intrauterine growth restriction	11 (8.7)	30 (11.5)	0.641
8	Anaemia	30 (23.8)	61 (23.4)	0.598
9	Failure to thrive	10 (7.9)	18 (6.9)	0.869
10	Diarrhoea	6 (4.8)	11 (4.2)	0.886
11	Hepatomegaly	47 (37.3)	99 (37.9)	0.905

[Table/Fig-3]: Comparison of the characteristic clinical symptoms of CMV-positive (n=126) and CMV-negative (n=261) subjects.
Data are presented as n (%), with comparisons made using the Chi-square test; p<0.05 indicates a significant difference

DISCUSSION

The current study highlights, for the first time, the difficulties in diagnosing possible congenital and postnatal CMV infection in symptomatic infants who present after 21 days of life. Routine newborn screening for CMV infection requires significant infrastructure and is unavailable in most resource-limited settings, such as ours. Thus, detection of CMV infection is possible only for newborns or young infants with compatible clinical manifestations. Unfortunately, even when such manifestations are present, the diagnosis is often missed due to low awareness of cCMV among healthcare professionals [22]. The duration of symptom onset is variable and may continue throughout infancy and beyond [4,23]. Thus, here we define a set of clinical criteria for screening CMV infection among infants in the absence of routine screening in resource-limited settings.

Among the total CMV-positive population (n=126), 6.35% (n=8) were confirmed cases of cCMV who presented within 21 days of life. The remaining 93.65% (n=118) of the confirmed cases presented after 21 days and were termed possible CMV infections. Thus, the majority of CMV-positive patients in present study population presented after 21 days of life. The Centers for Disease Control and Prevention (CDC) National Congenital CMV Disease Registry has defined possible CMV infection as the presence of signs and symptoms of cCMV that are detected from 21 days to one year of life [24].

When individual signs and symptoms were compared, none were significantly associated with CMV positivity. This is likely due to a lack of specificity of symptoms, a variable combination of symptoms in the individual patient, and variable appearance of symptoms with time [25-27]. As individual symptoms were not associated with the diagnosis of CMV infection, a COS for congenital CMV was framed. This constellation was formed with features characteristic of cCMV

	CMV positive	CMV negative	Total	Sensitivity (95% CI) %	Specificity (95% CI) %	Positive predictive value (95% CI) %	Negative predictive value (95% CI) %	Positive likelihood ratio (95% CI)	Negative likelihood ratio (95% CI)	p-value
Constellation of Symptoms; age group 1-365 days										
Present	99	157	256	78.57 (70.38, 85.38)	39.85 (33.86, 46.07)	38.72 (35.58,41.95)	79.36 (72.72, 84.72)	1.31 (1.14, 1.49)	0.54 (0.37, 0.78)	<0.001
Absent	27	104	131							
Total	126	261	387							
Constellation of Symptoms; age group 22-365 days										
Present	93	124	217	78.81 (70.33, 85.80)	41.78 (35.08, 48.72)	42.80 (39.24, 46.44)	78.11 (70.88, 83.95)	1.35 (1.17, 1.57)	0.51 (0.35, 0.74)	<0.001
Absent	25	89	114							
Total	118	213	331							
Constellation of Symptoms; age group 22-90 days										
Present	60	76	136	77.92 (67.02, 86.58)	40.62 (32.04, 49.66)	44.16 (39.63, 48.79)	75.33 (65.64, 83.00)	1.31 (1.09, 1.58)	0.54 (0.34, 0.87)	0.006
Absent	17	52	69							
Total	77	128	205							
Constellation of Symptoms; age group 91-365 days										
Present	33	48	81	80.49 (65.13, 91.18)	43.53 (32.80, 54.72)	40.70 (35.06, 46.59)	82.25 (70.39, 90.03)	1.43 (1.12, 1.81)	0.45 (0.23, 0.87)	0.008
Absent	8	37	45							
Total	41	85	126							

[Table/Fig-4]: Diagnostic accuracy measures of the Constellation of Symptoms (COS) among neonates and infants age-wise.
The COS includes cholestatic jaundice, hepatosplenomegaly, microcephaly and abnormality in neuroimaging mainly CNS calcification. Group comparisons in the contingency table were performed using the Chi-square test. p<0.05 indicates a significant difference. Diagnostic measures of the COS, including sensitivity, specificity, PPV, NPV, and positive and negative likelihood ratios, were calculated using MedCalc software.

Disease severity	Constellation Of Symptoms (COS)		p-value
Age group 1-365 days (Total CMV Positive cases, n=126			
Mild	Present (n=99)	Absent (n=27)	0.921
	32 (32.3)	9 (33.3)	
Moderate to severe	67 (67.7)	18 (66.7)	
Age group 22-365 days (Total CMV Positive cases, n=118)			
Mild	Present (n=93)	Absent (n=25)	0.882
	32 (34.4)	9 (36.0)	
Moderate to severe	61 (65.6)	16 (64.0)	
Age group 22-90 days (Total CMV Positive cases, n=77)			
Mild	Present (n=60)	Absent (n=17)	0.500
	23 (38.3)	5 (29.4)	
Moderate to severe	37 (61.7)	12 (70.6)	
Age group 91-365 days (Total CMV Positive cases, n=41)			
Mild	Present (n=33)	Absent (n=8)	0.215
	9 (27.3)	4 (50.0)	
Moderate to severe	24 (72.7)	4 (50.0)	

[Table/Fig-5]: Relationship of disease severity with a Constellation Of Symptoms (COS) used in clinical screening.
Data are presented as n (%), with comparisons made using the Chi-square test in different age groups; p<0.05 indicates a significant difference

infection and relatively easy to detect by clinical examination and ultrasonography. The presence of one or more features of the constellation in a study subject was taken as the criterion for clinical screening of CMV infection, and subjects satisfying this criterion were selected for PCR testing. The presence of COS was significant in the CMV-positive group (p-value<0.001). The presence of COS with CMV infection was also significant when the infants were stratified into different age groups. The majority of the CMV-suspected patients presented up to 90 days (n=205), while the number of CMV-suspected patients decreased from 91 to 365 days (n=126).

Present study evaluated the diagnostic accuracy measures to validate this screening strategy for CMV infection. The sensitivity was reasonably high (77-81%), while the specificity was low (40-44%). As the sensitivity of the COS is acceptable, this constellation may be used as a screening tool to determine whether CMV PCR testing is warranted. This approach can reduce the need for PCR testing, which may not be readily accessible in resource-limited settings. Given the low specificity, the presence of COS cannot be used as a confirmatory diagnosis. The performance of COS is supported by the PPV and NPV, which ranged from 38% to 45% and 75% to 83%, respectively. Although the PPV is low, the NPV is reasonably high. However, even with a favourable NPV, about 20% of CMV infections could be missed by COS screening; consequently, PCR testing would be used as a confirmatory test to identify the true CMV-infected cases among those screened. The remaining 20% of missed cases would require clinical follow-up. Two scenarios may arise: either the infant's symptoms subside, in which case the infection is not life-threatening, or the symptoms persist, in which case a confirmatory PCR test should be performed to rule out CMV infection. The issue of false positives is mitigated because COS is intended for screening only. Therefore, the COS strategy should be applied only in settings where routine screening is not practised. Overall, the COS strategy reduces the number of PCR tests and should sensitise clinicians to request CMV testing. A possible reason for the relatively low diagnostic values is the non specificity of the signs and symptoms of CMV infection [28,29]. Cholestatic jaundice and hepatosplenomegaly were included in the COS because they are common clinical presentations of cCMV infection. In the newborn period, there are many causes of these symptoms, namely congenital infections other than CMV, metabolic disorders, and idiopathic hepatitis [30]. CMV disease were categorised by severity and investigated the relationship between COS and disease severity by categorising infants into different age groups. The presence of COS was not significantly different between patients with mild and those with moderate-to-severe CMV disease in any age group. Thus, the COS helps in detecting CMV infection, but it is not instrumental in differentiating disease severity.

Limitation(s)

There are several notable limitations to present study. First, although the sensitivity of the COS is within an acceptable range, its use is likely to miss approximately 20% of CMV cases. This reflects the operational constraints of healthcare systems in many developing countries, where routine universal screening for cCMV is not feasible due to limited resources and infants often present only after becoming symptomatic. Therefore, COS should be applied only in settings where routine screening is not available. Second, since present study population included only symptomatic CMV-suspected patients, the current screening strategy detects CMV infection only among symptomatic patients up to 365 days of life.

CONCLUSION(S)

In conclusion, present study highlights a clinical screening approach for CMV infection in resource-limited settings beyond 21 days and the challenges involved. Compared with patients infected with cCMV, a greater proportion of CMV-infected patients in present cohort—those with possible CMV infection including both congenital and postnatal CMV—presented after 21 days. No solitary signs or symptoms correlated with CMV positivity. The COS is instrumental in screening for possible CMV-infected patients and serves as a reasonably informative diagnostic tool in resource-limited regions of developing countries. The proposed COS screening showed efficacy up to 365 days of age in detecting CMV infection.

Acknowledgement

Authors gratefully acknowledge the authorities of National Institute of Cholera and Enteric Diseases for supplying this study's essential resources and instrumentation. Authors are also grateful to all the participants and their guardians.

Authors' contributions: SA conceptualised and designed the study, performed the experiments, searched the literature, extracted and analysed the data, and drafted the initial paper. SKG extracted and analysed the data, and critically reviewed and revised the paper. RPC performed the experiments. KC, SG performed the data analysis. MN and BB critically reviewed and revised the paper. NC conceptualised and designed the study, and critically reviewed and revised the paper. All authors read and approved the final paper as submitted.

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PLAGIARISM CHECKING METHODS: [Jain H et al.]
- Plagiarism X-checker: May 13, 2025
- Manual Googling: Aug 02, 2025
- iThenticate Software: Aug 05, 2025 (8%)

ETYMOLOGY: Author Origin

EMENDATIONS: 7

AUTHOR DECLARATION:
- Financial or Other Competing Interests: None
- Was Ethics Committee Approval obtained for this study? Yes
- Was informed consent obtained from the subjects involved in the study? Yes
- For any images presented appropriate consent has been obtained from the subjects. NA

Date of Submission: **May 03, 2025**
Date of Peer Review: **Jun 02, 2025**
Date of Acceptance: **Aug 07, 2025**
Date of Publishing: **Nov 01, 2025**