EDITORIAL

Clinical Trial Registry - India (CTR-I): A meaningful initiative. How to take it forward?
Bavdekar SB

ORIGINAL ARTICLES

Detection of Rh antibodies using two low ionic diluents: Extension of the incubation time and the number of Rh antibodies detected
Skaik YA

Immunophenotypic characterisation of peripheral T lymphocytes in pulmonary tuberculosis
Al M ajid FM, Abba AA

Relationship between N-terminal pro-B type natriuretic peptide and extensive echocardiographic parameters in mild to moderate aortic stenosis
Cemri M, Arslan U, Kocaman SA, Çengel A

Relative efficiency of polymerase chain reaction and enzyme-linked immunosorbent assay in determination of viral etiology in congenital cataract in infants
Shyamala G, Sowmya P, M adhavan H N, M alathi J

Stomaplasty—anterior advancement flap and lateral splaying of trachea, a simple and effective technique
Trivedi NP, Patel D, T hankappan K, Iyer S, Kuriakose M A

CASE REPORTS

Rhodotorula mucilaginosa as a cause of persistent femoral nonunion
Goyal R, D as S, Arora A, Aggarwal A

Repeated fracture of pacemaker leads with migration into the pulmonary circulation and temporary pacemaker wire insertion via the azygous vein
U dyavar AR, Pandurangi U M, Latchumanadhas K, M ullasari AS

Recurrent respiratory papillomatosis complicated by aspergillosis: A case report with review of literature
Kuruvilla S, Saldanha R, Joseph L D

Citrobacter freundii infection in glutaric aciduria type 1: Adding insult to injury
M ukhopadhyay C, D ey A, Bairy I

IMAGES IN RADIOLOGY

Chordoma: A rare presentation as solitary ivory vertebra
Kumar S, Has an R

IMAGES IN PATHOLOGY

Intracystic papillary carcinoma associated with ductal carcinoma in situ in a male breast
Dragoumis D M, Tsiftsoglou AP
REVIEW ARTICLE
Implications of HLA sequence-based typing in transplantation
Shankarkumar U, Pawar A, Ghosh K 41

DRUG REVIEW
Ramelteon: A melatonin receptor agonist for the treatment of insomnia
Devi V, Shankar PK 45

STUDENTS CORNER
The internet: Revolutionizing medical research for novices and virtuosos alike
Jethwani KS, Chandwani HS 49

VIEW POINT
Documenting indications for cesarean deliveries
Kushtagi P, Guruvare S 52

CLINICAL SIGNS
Cherry-red spot
Suvarna JC, Hajela SA 54

LETTERS
Central retinal vein occlusion associated with thrombotic thrombocytopenic purpura/hemolytic uremic syndrome
Author’s reply 58
Simultaneous umbilical hernia repair in patients undergoing laparoscopic cholecystectomy: Is obesity a risk factor for recurrence?
Authors’ reply 59
Snap sound and detumescence: Fracture penis
Paraphenylene diamine-induced acute renal failure: Prevention is the key
Inadequate awareness of the role of erythrocytic parameters in the detection of beta-thalassemia minor
Model for end-stage liver disease and outcome of portosystemic encephalopathy
Aortic thrombus during invasive aspergillosis in a kidney transplant recipient
Castleman’s disease in interpectoral lymph node mimicking mammary gland neoplasia
Bacterial endocarditis due to Group C streptococcus
Postpartum Group B streptococcal meningitis

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Editorial Office
Department of Pediatrics,
Seth GS Medical College and KEM Hospital,
Parel, Mumbai 400012, India.
E-mail: jgpm@jgmonline.com

Administrative Office
Medknow Publications,
A-108/109 Kanara Business Centre, Ghantokar (E), Mumbai - 400075, India.
Phone: 91-22-6649 1818/1816
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Relative efficiency of polymerase chain reaction and enzyme-linked immunosorbant assay in determination of viral etiology in congenital cataract in infants

Shyamala G, Sowmya P, Madhavan HN, Malathi J

ABSTRACT

Background: Perinatal viral infections of fetus are among the leading causes of congenital cataract and identifying the viral etiology is important. Objectives: To detect the presence of Rubella virus (RV), herpes simplex virus (HSV) and cytomegalovirus (CMV) in lens aspirate specimens obtained from patients with congenital cataract and relate the results with serology. Setting and Design: Prospective study carried out in tertiary care hospital. Materials and Methods: Fifty lens aspirates from 50 infants with congenital cataract were subjected to HSV, RV isolation and polymerase chain reaction (PCR) for detection of HSV and CMV. Reverse transcription polymerase chain reaction (RT-PCR) was applied for RV detection. Peripheral blood specimens were screened for anti-HSV, RV and CMV antibodies by enzyme-linked immunosorbant assay (ELISA). Results: Rubella virus was detected in nine (18%) lens aspirates, by nRT-PCR which includes six positive by culture. HSV-2 DNA was detected in nine other lens aspirates, while CMV was not detected by PCR. Serological results did not correlate with the presence of viruses in the lens aspirates. This is the first report of detection of HSV-2 DNA in cases of congenital cataract. Conclusions: Cytomegalovirus may not be playing a significant role in causation of congenital cataract. The role of serology in identifying causative viral infection for congenital cataract needs to be re-evaluated.

KEY WORDS: Intrauterine infection, ocular manifestations
No attempt was made to isolate CMV due to nonavailability of human diploid fibroblast cell lines. The isolation of a particular virus was confirmed by either doing a semi-nested polymerase chain reaction (snPCR) for HSV or by nested reverse transcription polymerase chain reaction (nRT-PCR) for RV.

Serum samples from all 50 patients were collected at varying periods of 2 days to 3 months prior to surgery and tested for the presence of IgG and IgM antibodies against RV, HSV and CMV by commercial ELISA kits (Biokit SA, Barcelona, Spain) as per the manufacturer’s instructions. Serum samples of the patients were diluted 1:10 for the detection of anti-RV, HSV and CMV IgG antibodies and anti-RV, HSV and CMV IgM antibodies. The results were read at 490 nm in an ELISA plate reader (Bio-Tek Model EL-311, USA or Dynatech, USA) and expressed as ELISA units (EU) calculated based on the OD values of appropriate controls.

RNA was extracted from lens aspirate specimens using Qiagen Viral RNA extraction kit (Cat no. 52904) procured from Qiagen, Hilden, Germany according to the manufacturer’s instructions. RNA was finally eluted in 60 µL of RNase-free water and immediately frozen at -80°C. The extracted RNA was required for detection of RV by nRT-PCR.

DNA was extracted from 100 µL of the diluted lens aspirate specimen using the clinical genomic DNA mini prep kit (Biogene Inc, CA, USA) as per the manufacturer’s instructions. The extracted DNA was used for snPCR for HSV and nested PCR (nPCR) for CMV.

Herpes simplex virus from clinical specimen was detected by application of snPCR with primers flanking the glycoprotein D gene of the HSV (1 and 2) genome.[7] The snPCR protocol described earlier by us was followed.[8] All the reagents used for PCR were procured from Bangalore Genei Pvt. Ltd, India. All the reactions were carried out using appropriate negative control which contained PCR reagents without DNA (deionized water was added to make up the volume) and positive control. DNA samples extracted from respective standard strains of HSV-1 and HSV-2 was used as the positive controls. This standardized snPCR technique was used for the detection of HSV DNA in the 50 lens aspirate samples. The presence of 272 bp in the second round of amplification was taken to indicate the presence of HSV DNA. Although both HSV 1 and 2 produce the same base product size, the primers used in this study were specific for HSV 2, hence it could be inferred that the amplified DNA was that of HSV 2.

The nPCR targeting the morphological transforming region II of CMV standardized earlier by us was applied for the detection of the same; the sensitivity and specificity of the PCR have also been reported by us.[9] The presence of a 168 bp-sized product was taken to indicate the presence of CMV DNA.

Rubella virus cDNA was generated using a one step RT-PCR kit (Qiagen, Germany). The reaction was performed in 50 µL volumes per the manufacturer’s instructions. In brief, the reaction consisted of 400 µM of each dNTP, 1× buffer, 0.6 µM of each R1 and R2 primers and the enzyme mix. The primer sequences for the I round were R1 - 5’ CAA CAC GCC GCA CGG ACA AC 3’ and R2 - 5’ CCA AAA GCC GCC CGG AGC AGT CA 3’ and R3 - 5’ CTC GAG GTC CAG GTC CAG CTG CTG CC 3’ and R4 - 5’ GAA TGG CGT TGG CAA ACC GC 3’[10] for the I round. The enzyme mix consisted of both the Omniscript and Sensiscript reverse Transcriptase and (hot start) Taq DNA polymerase in 1 unit. These enzymes being recombinant heterodimeric enzymes expressed in Escherichia coli, they exhibit higher affinity for RNA, facilitating transcription through secondary structures that inhibit other reverse transcriptases.

The enzyme mix facilitated both reverse transcription and PCR. The reaction mix was incubated in the thermal cycler thus: 50°C for 30 min for reverse transcription followed by the activation of DNA polymerase enzyme and inactivation of omniscript and sensiscript reverse transcriptases at 95°C for 15 min. The PCR amplification then proceeded for 40 cycles, by denaturing the cDNA template at 94°C, annealing at 60°C for 30 s and extension at 72°C for 1 min. The final extension was carried out at 72°C for 5 min. For the nested amplification, 2 µL of the first round product was added to 50 µL of the PCR mix consisting of 200 µM of each dNTP 10 mM Tris-Cl, 0.6 µM of R3 and R4 primers and 2.5 U of Taq DNA polymerase. The amplification was performed in the same way as in the first round except that only 25 cycles were used. Ten micro litres of the amplified DNA product were resolved by 2% ethidium bromide agarose gel electrophoresis and visualized in UV transilluminator. Amplification of 143 bp produced in nested PCR indicated the presence of rubella-specific RNA. Each PCR was run with specific positive and negative controls. Negative controls used were the uninfected Vero cells and the positive control was the HPV77 standard strain of RV (procured from National Institute of Virology (NIV), Pune). All PCR amplifications were carried out using PCR thermal cycler PE Applied Biosystems 2700, USA.

In order to prevent DNA/RNA contamination, the extraction of DNA, setting up of PCR reactions and amplification of PCR, loading of the gel each were carried out in physically separate rooms. Filter guarded tips were used to prevent the contamination of pipettes. For addition of positive control, separate pipette fixed with filter guarded tips was used.

Results

Rubella virus was isolated from six (12%) lens aspirates using the SIRC cell line and three of these were also isolated in the Vero cell line, which was confirmed by detection of RV RNA in nRT-PCR. Herpes simplex virus was not isolated from any of the lens aspirates. The nRT-PCR for E1 gene was detected in nine (18%) lens aspirate specimens, which included the six specimens positive for culture [Table 1]. The HSV DNA was detected in nine (18%) specimens. All nine strains were identified as HSV 2. The optimized nPCR did not detect CMV DNA in any of the specimens.

Comparative results of PCR and serological tests for the presence of IgG and IgM antibodies against RV, HSV in 18
infants positive for either of the viruses are depicted in Table 1. Anti-CMV IgM antibodies were not detected in any of the samples while anti-CMV IgG antibodies were detected in 27 specimens.

Discussion

Based on virus isolation and serology, RV has been reported to be associated with 10% of cases of congenital cataract in a hospital-based study. This study used nRT-PCR for the detection of RV RNA for improving sensitivity and documented that up to 18% of congenital cataracts are associated with RV. Direct evidence of association of RV and HSV 2 were demonstrated in 18 (36%) lens aspirates with RV in nine (18%) and HSV 2 in another 9 (18%) aspirates. Rubella virus was isolated in tissue culture from six of the nine RV-associated lens aspirates. This probably is the first report from India that has recorded an association of these viruses with congenital cataract.

In most such studies done in India and elsewhere, only serology has been used to demonstrate for such associations. As serological studies present only indirect evidence, these results should be carefully interpreted. Anti-RV IgM antibodies were present in the serum samples of only three of the nine patients whose lens aspirates demonstrated RV RNA. This suggests that the absence of anti-RV IgM antibodies does not necessarily exclude a diagnosis of congenital rubella syndrome. It is possible that early primary infection of the lens by RV, when the fetal immunological apparatus was insufficiently mature to react to the viral antigen was responsible for absence of antibodies. It has been noted that lenses from fetuses that had been infected in the first trimester by the RV exhibit pyknotic nuclei, cytoplasmic vacuoles and inclusion bodies in the primary lens cells and retardation of lens development; late changes included degeneration of some primary lens fibres and evidence of active disease in the newly developing equatorial lens fibre cells, indicating chronic infection. Direct detection of viral RNA in clinical samples can be expected to identify almost all cases of intrauterine RV infection within 24–48 h after sampling. In the present study, we have shown that nRT PCR is a more sensitive and rapid technique than the conventional method of virus isolation and serology for the diagnosis of congenital rubella syndrome.

Even in case of HSV infection, nine lens aspirates showed the presence of HSV 2 DNA, although anti-HSV-2 antibodies were not detected in the serum samples. Cibis et al. have suggested that in congenital cataract due to HSV, the virus possibly enters the lens during the first trimester and persists throughout the period of fetal development and hence is considered a ‘self-antigen’ by the fetal immune system. Newborns typically acquire HSV-2 infection during passage through an infected birth canal, but transplacental spread does account for a small proportion of cases. Although our results correlate well with the results of studies performed in the early 1970s and 1980s, a recent study by Raghu et al., reported the association of HSV-1 DNA with congenital cataract. Some studies suggest that cataracts not only progress, but may also develop after birth. Failure to isolate HSV-2 from the lens aspirates of any of the 9 HSV-2 DNA-positive patients, suggests the absence of active viral replication in the infected infants. The virus had possibly left an imprint of its DNA during its infection of the lens which led to congenital cataract, before becoming inactive.
The results of our study suggest that serology has little or no role to play in identifying the causative virus in congenital cataract. Most of our patients showed IgG type antibodies against the three viruses studied and these were probably acquired through the transplacental transfer. Although CMV is said to be one of the commonest causes of congenital infections worldwide, the results of the present study suggest that this virus had no role to play in the development of cataract in the 50 patients investigated. Serology indicated the presence of anti-CMV IgG in 27 patients and this was possibly due to passive transfer of antibodies from the mother through the placenta.

We conclude that nucleic acid amplification tests such as PCR may be of value in confirming the etiology of congenitally acquired infection of the lens. Serology appears to play little or no role in identifying the pathogen. The fact that HSV-2 DNA was detected in nine lens aspirates which did not reveal CMV or RV, while RV RNA was detected in another nine aspirates which did not reveal HSV-2 or CMV, suggests that HSV-2 might be playing an important role in causing congenital cataract. Conversely, CMV appears to have a limited role in the causation of congenital cataract in the context of patients presenting to our hospital.

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