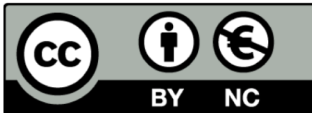

Main Article

Comparison between ELISA and PCR for Detection of Epstein-Barr Virus in Children with Acute Cervical Lymphadenitis

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ABSTRACT

Introduction

Among viruses, Epstein Barr Virus is an important cause of acute cervical lymphadenitis. The clinical features of acute Epstein-Barr virus infection overlap those of a variety of other infectious and non-infectious diseases so reliable laboratory tests are important to not only confirm but aid in the differential diagnosis. The aim of the present study was to investigate and compare the diagnostic utility of EBV DNA detection as an adjunct to serological diagnosis of primary EBV infection.

Materials and Methods

In this hospital based cross sectional study 30 patients below 18 years of age with acute cervical lymphadenitis were included. Comparison between ELISA and PCR for detection of Epstein - Barr virus in children with acute cervical lymphadenitis was done. EBV IgM and IgG-ELISA was done for the qualitative determination of IgM and IgG class antibodies against Epstein-Barr virus Viral Capsid Antigen (VCA) in serum samples and Real-time PCR (by using RTP Pathogen Kit) was done for qualitative determination of EBV DNA.

Results

EBV RT-PCR was negative in serum samples of all 30 patients. EB virus could not be detected by RT-PCR in all seropositive cases. There was no significant difference found between genders in the results of EB Virus serology, and that of EBV RT PCR (p value was >.05).

Conclusion

The use of RT- PCR for EBV DNA detection resulted in an increase in reliability of diagnosis of primary EBV infection, enhancing overall diagnostic efficacy. Our study proves that serological antibody could be positive for EBV in many patients either because of cross reactivity with other viruses or past infection with EBV.

Keywords

Epstein Barr Virus; EBV IgM; PCR; EBV DNA; Cervical Lymphadenopathy

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Acute cervical lymphadenitis is frequently encountered by otorhinolaryngologists in day-to-day practice. Cervical lymphadenitis is defined as an acute symptomatic enlargement of the cervical lymph nodes. Lymphadenitis particularly refers to lymphadenopathies that are caused by inflammatory process. However, the terms lymphadenitis and lymphadenopathy are often used interchangeably. Most common etiologies of lymphadenopathy are infections,

tuberculosis, neoplasia, autoimmune diseases, iatrogenic causes and miscellaneous conditions[1].

In paediatric population cervical lymphadenitis is a common problem. According to a study cervical lymphadenopathy affects around 90% of children aged 4 to 8 years.[2] In the paediatric population, most common cause of lymphadenopathies is infectious aetiology.[3] Acute viral lymphadenitis is the most common form of reactive lymphadenitis and typically develops following URTI. The common viruses involved are Epstein-Barr virus, Adenovirus, Cytomegalovirus, Rhinovirus, Coxsackie virus A and B, Parainfluenza, & Influenza virus.[4] Viral upper respiratory tract infection usually causes acute bilateral cervical lymphadenitis. Pyogenic bacteria such as staphylococcus aureus or streptococcus usually causes acute unilateral cervical lymphadenitis.[5]

Among viruses, Epstein Barr Virus is an important cause of acute cervical lymphadenitis. Epstein-Barr virus (EBV) was first discovered in year 1964 by Epstein and Barr in cells isolated from Burkitt's lymphoma. EBV infection is highly prevalent worldwide, with more than 90% of the adults being infected with the virus. EBV is a member of the herpes virus family (HHV4) and is a double stranded DNA virus. There are two types of EBV, EBV-1and EBV-2. The oral route is the primary route of the EBV transmission. However, it has been reported that organ transplantation and blood transfusion can lead to EBV spread.

Most of the primary EBV infections in children are usually asymptomatic.[6] Most common acute presentation of EBV infection is a febrile viral upper respiratory illness. Similar to other herpes viruses, following a primary infection, the EBV has a latency phase where it infects epithelial cells, enters the circulating B lymphocyte, and persists for the life in a latent state.[7] The incubation period of symptomatic primary EBV infection is about six weeks. EBV infection is associated with the many diseases, including infectious mononucleosis, Burkitt's lymphoma, Hodgkin lymphoma, nasopharyngeal carcinoma.[8] In addition, this virus has been linked to a wide range of diseases such as post transplant lymphoproliferative diseases, gastric carcinoma, systemic lupus erythematosus, chronic fatigue syndrome, rheumatoid arthritis, thyroid disorders, multiple sclerosis and other autoimmune disorders.[9]

The clinical features of acute Epstein-Barr virus infection overlap those of a variety of other infectious and non-infectious diseases, and reliable laboratory tests are important to not only confirm but aid in the differential diagnosis.[10] Primary EBV infection can be diagnosed by viral DNA in cell-free serum or plasma of patient or by an assay for heterophile antibodies. For indirect evidence serological studies can demonstrates the presence of IgM antibodies in acute infection and IgG antibodies for chronic infection against different EBV antigens. During early primary infection, IgM class of antibody to Epstein Barr viral capsid antigen is detected; whereas detection of IgG to viral capsid antigen is seen in past infections. Both IgM and IgG antibodies are detected during recent infection or reactivation.[5] EBV infection usually confirmed by clinical manifestation and EBV serology. Furthermore, serological studies are often done but they are relatively unreliable and insensitive for diagnosis. Heterophile antibodies assay's sensitivity is low for children. In primary infection of children, the appearance of EBNA antibodies may be unusually delayed, resulting in a false diagnosis of recent EBV infection. Therefore, Viral DNA in cell-free serum or plasma of patients is most useful direct evidence for diagnosing primary EBV infection.[11]

American scientist Kary Mullis devised PCR in 1983. Qualitative PCR is used to determine the presence and absence of specific DNA product. It is used for cloning or pathogen identification. Quantitative real time PCR allows real time measurement and provides information far beyond the DNA detection. It shows how much of specific DNA is present in the sample. Real-time PCR is a more rapid, sensitive, specific and reproducible method for detecting and monitoring the levels of EBV in comparison to conventional PCR.

The lack of knowledge about immune system response to EBV has impaired our ability to develop an effective prophylactic vaccine against EBV. Greatest challenge in recent time is to develop a prophylactic vaccine and devise treatment strategies for EBV infected patient.[12] The Timely diagnosis will definitely help clinicians to avoid a

battery of investigations and misuse of antibiotics. In India there are very limited studies available that have employed qualitative RT-PCR for laboratory evidence of Epstein Barr virus associated acute cervical lymphadenitis in paediatric patients.

Materials and Methods

This hospital based cross sectional study was conducted in the Department of ENT& Microbiology during the period from September 2018 to April 2020. All patients in the age below 18 years from ENT OPD were enrolled for the study. Exclusion criteria include neck swelling other than lymph node origin. Known case of lymphadenopathy due to tuberculosis and malignancy, which were on treatment, were excluded from the study. Parents of children were informed about the study. Written and informed consent was given by the parents. The study protocol was approved by the institutional ethics committee. A detailed and relevant history was taken followed by a thorough general physical, ENT and Head & neck examination. Necks of patients were thoroughly examined for any palpable lymph nodes. Patient's blood sample was taken in plain vials and serum was separated by centrifugation at room temperature. Serum samples were stored on ice during transportation and serum samples were stored in deep freeze. Multiple thawing and freezing was avoided before isolating the viral DNA. All 30 serum sample were subjected to RT-PCR for detection of Epstein-Barr virus as per the RTP® Pathogen Kit manufacturer guidelines. Isolation of DNA of EBV was done by Sample lysis, binding of the DNA, first washing of the RTA Spin Filter, second washing of the RTA Spin Filter and elution of the DNA. The eluted DNA was used in different subsequent applications. Samples of heparinised patients were not used as heparin is a PCR interfering substance. The viral DNA was amplified by polymerase chain reaction. The presence of specific pathogen sequences in the reaction was detected by an increase in fluorescence observed from the relevant dual-labelled probes, and was reported as a cycle threshold value (Ct) by the Real-time thermo cycler. Results were analyzed according to Real-time PCR kit manufacturer guidelines.

Epstein - Barr virus IgM and IgG-ELISA was intended for the qualitative determination of IgM and IgG class antibodies against Epstein-Barr virus viral capsid antigen in human serum. Before assaying, all samples were diluted 1 in 100 with IgM and IgG sample diluent.10ul sample dispensed with 1ml IgM sample diluents into the tubes to obtain a 1 in 100 dilution. ELISA microwell plate reader was adjusted to zero using the substrate blank absorbance of the wells measured at 450nm and the absorbance values recorded for each standard and sample in the plate layout. Results were analyzed according to ELISA kit manufacturer guidelines and statistical analysis was done.

Categorical variables were presented in number and percentage (%) and continuous variables were presented as mean ± SD and median. Qualitative variables were compared using Chi square test/Fisher's exact test. A p value <.05 was considered as statistically significant. The data was entered in MS EXCEL spreadsheet and analysis was done using Statistical Package for Social Sciences (SPSS) version 21.0.

Results

Our study was conducted in the Department of Otorhinolaryngology & Microbiology department of Vardhman Mahavir Medical College & Safdarjung Hospital, New Delhi during the period of September 2018 to April 2020.Thirty(30) children below 18 years of age with acute cervical lymphadenitis were included in the study and results are as follows.

Table I : Age Distribution

AGE (YEARS)	FREQUENCY	PERCENTAGE
1-5 years	12	40.00%
6-10 years	11	36.67%
>10 years	7	23.33%
Mean ± SD	6.97 ± 3.1	
Median (IQR)	6 (5-8.75)	
Range	2-13	

In present study, 40% of patients belonged to age group 1-5 years followed by 6-10 years (36.67%). Age was >10 years in only 7 out of 30 patients. Mean value of age (years) of study subjects was 6.97 ± 3.1 with median (IQR) of 6 (5-8.75). (Table I)

Sixty three percent (approx.) (63.33%) of patients were males and 11 out of 30 patients were females.

26 (86.67%) patients had bilateral lymph nodes. Only 4 out of 30 patients had unilateral lymph nodes. In majority (90%) of patients, tenderness was present. Tenderness was absent in only 3 out of 30 patients. In all thirty 30(100%) patients, level of lymph node was II followed by level I (83.33%), level III (16.67%) and level V (10.00%). Level of lymph node was IV in none of the patient.

Sixty three percent (63.33%) of patients, haemoglobin was 11.1-13 gm/dl followed by >13 gm/dl (16.67%) and <9 gm/dl (13.33%). Haemoglobin was 9-11 gm/dl in only 2 out of 30 patients.

Fifty six percent (56.67%) of patients, total leucocyte count was 12100/mm^3-14000/mm^3 followed by 10000/mm3-12000/mm^3 (16.67%). Total leucocyte count was <10000/mm^3 and >14000/mm^3 in only 4 out of 30 patients each. (Table II)

In majority (96.67%) of patients, EBV IgM was negative. EBV IgM was positive in only 1 out of 30 patients. (Table III)

Table II : Distribution of total leucocyte count of study subjects

TOTAL LEUCOCYTE COUNT	FREQUENCY	PERCENTAGE
<10000/mm^3	4	13.33%
10000-12000/mm^3	5	16.67%
12100-14000/mm^3	17	56.67%
>14000/mm^3	4	13.33%
Total	30	100.00%

Table III : Distribution of EBV IgM of study subjects.

EBV IgM	FREQUENCY	PERCENTAGE
Negative	29	96.67%
Positive	1	3.33%
Total	30	100.00%

Table IV: Comparison of EBV IgM between genders

EBV IgM	FEMALE (N=11)	MALE (N=19)	TOTAL	P VALUE	TEST PERFORMED
Negative	11 (100%)	18 (94.74%)	29 (96.67%)	1	Fisher Exact test
Positive	0 (0%)	1 (5.26%)	1 (3.33%)		
Total	11 (100%)	19 (100%)	30 (100%)		

No significant gender difference was seen in the distribution of EBV IgM. (P value>.05) EBV IgM was negative in 100% in female and 94.74% in male and positive in 0% of patients in female and 5.26% of patients in male with no significant difference between them. (Table IV).

In present study, in majority (90.00%) of patients, EBV IgG was positive. EBV IgG was negative in only 3 out of 30 patients. (Table V)

No significant gender difference was seen in the distribution of EBV IgG.

Table V : Distribution of EBV IgG of study subjects

EBV IgG	FREQUENCY	PERCENTAGE
Negative	3	10.00%
Positive	27	90.00%
Total	30	100.00%

(p value>.05) EBV IgG was positive in 100% in female and 84.21% in male and negative in 0% of patients in female and 15.79% of patients in male with no significant difference between them. (Table VI)

Table VI: Comparison of EBV IgG between genders

EBV IgG	FEMALE (N=11)	MALE (N=19)	TOTAL	P VALUE	TEST PERFORMED
Negative	0 (0%)	3 (15.79%)	3 (10%)	0.279	Fisher Exact test
Positive	11 (100%)	16 (84.21%)	27 (90%)		
Total	11 (100%)	19 (100%)	30 (100%)		

Table VII : Distribution of EBV RT PCR of study subjects

EBV RT PCR	FREQUENCY	PERCENTAGE
Negative	30	100.00%
Total	30	100.00%

In present study, in all the patients, EBV RT PCR was negative. (Table VII)

In present study all patients were EBV RT PCR negative. (Table VIII)

Table VIII: Comparison of EBV RT PCR between genders

EBV IgG	FEMALE (N=11)	MALE (N=19)	TOTAL	P VALUE	TEST PERFORMED
Negative	11 (100%)	19 (100%)	30 (100%)	No p value	-
Total	11 (100%)	19 (100%)	30 (100%)		

Discussion

Our study, a hospital based cross sectional study was conducted in the Department of Otorhinolaryngology & Microbiology of a Medical College & Hospital, New Delhi during the period of September 2018 to April 2020. Thirty children below 18 years of age with acute cervical lymphadenitis were included in the study. All patients were analysed with respect to age, sex, laterality, level of lymph node, tenderness, consistency, haemoglobin, total leukocyte count, Epstein Barr virus IgM and IgG and RT PCR for EBV.

A cross-sectional study by Jalal et al[6] conducted among eighty two children presented with cervical lymphadenopathy out of which Epstein-Barr virus infection was diagnosed in 13 (15.9%) children (n=82). The average age of these cases was 7.5 (SD±3.3) years with 1.6 male to female ratio. The anterior group cervical lymph nodes were commonly affected. On examination lymph nodes was mobile and firm in majority, 12 (92.3%) of patient. The lymph node number ranged from 2-6 nodes; with a mean of 3.5 nodes. Increase in total leukocyte count was seen in 3 patients.

Our study was a hospital based cross sectional study, conducted among 30 children presented with cervical lymphadenopathy out of which EBV infection was diagnosed in 27 (90%) children (n=30). A cross-sectional study by Jalal et al[6] conducted among eighty two children presented with cervical lymphadenopathy out of which Epstein-Barr virus infection was diagnosed in 13 (15.9%) children (n=82).

In our study, the average age of children was 6.97 ± 3.1 with median (IQR) of 6(5-8.75). The male to female ratio was 1.7. These observations are in accordance with that of Jalal et al. where the average age of these cases was 7.5 (SD±3.3) years with 1.6 male to female ratio.

Cervical group of lymph nodes was seen in majority (90%) of patient. On examination, majority of patient had mobile and firm lymph node. These results in this study are similar to other studies. Increase in total leukocyte

was found in 21(70%) of the patient. In an Indian Retrospective observational study, Nandi et al[10] studied clinical and laboratory features of fifty-three (53) serologically EBV positive infectious mononucleosis children aged between 1 month to 12 years. The majorities (89.5%) cases aged between 5 and 8 years were found to have cervical lymphadenopathy. Similarly, Balfour et al[13] observed in 95%, Grotto et al[14] observed in 88.0%, and Gao et al[15] observed in 89.5% of the cases.

Sarsu et al[16] studied population consisted of 1003 (59%) boys and 697 (41%) girls aged less than 18 years. Forty-three patients with unilateral and 452 patients with bilateral lymphadenopathies were studied. On conclusion, most widely encountered cause of lymphadenopathy was cytomegalovirus (CMV) and Epstein-Barr virus (EBV) infections. Similarly in present study, we observed twenty-seven (27) (90%) patients with cervical lymphadenopathy who were positive for Anti VCA- IgG antibody. That means cases included in study acquired this infection in their lifetime. EBV IgG was positive in 100% in female and 84.21% in male, no significant difference was seen in the distribution of EBV IgG between genders (p value>.05).

Figueira et al[17] studied the prevalence of EBV antibodies in a sample of 283 children and adolescents between 1 and 21 years old. Anti-VCA (Virus Capsid Antigen) IgG antibodies were detected in 71% of patients by ELISA kits. These results demonstrate that there is a high prevalence of EBV antibodies occurs more frequently at a younger age in children from families with low socioeconomic status. Similarly in present study, Anti-VCA (Virus Capsid Antigen) IgG antibodies against EBV were detected in 90% of patients.

In a study done for serological diagnosis of primary EBV infection and EBV reactivation with real-time EBV PCR by Luderer et al[18] the diagnosis of primary EBV infection was established for 24 of the 45 IgM VCA-positive patients. Out of 24 IgM positive case of primary infection, no EBV DNA was detected in five cases by PCR. EBV DNA was detected only in two of the 62 serum sample of reactivation profile cases.

In a study Paschale et al[19] found that, EBV DNA could be determined in serum or plasma. In patients with primary infection, it is frequently detected in whole blood (plasma/serum) within 14 days of symptoms onset. After the initiation of an immune response, viral load decreases slowly in peripheral blood mononuclear cells, but rapidly in plasma/serum, and it becomes undetectable after 3-4 weeks. Viral load may increase after an initial decline, and in some cases, it may take as long as a year or more before it reaches stably low levels. Finally, even when this level is reached, the blood of a healthy carrier contains 1-50 copies of EBV DNA per million white blood cells, whereas EBV-DNA is almost always undetectable in plasma or serum.

In a study Gartzonika et al[20] collected serum sample from 118 patients of suspected primary EBV infection. After serological testing, a quantitative real time EBV PCR assay was performed. Samples which were drawn 20 days after onset of symptoms were negative and younger patients were found to have higher viral load. In a study Elansary et al[21] found 38 patients reactive for anti-VCA antibody against EBV and PCR was negative for all of the reactive cases. In present study, EBV RT PCR was negative in all twenty-seven (27) seropositive cases.

Conclusion

RT-PCR for EBV was negative in serum samples of all thirty (30) patients. EB virus could not be detected by RT-PCR in all seropositive cases. However, a firm diagnosis cannot be made with serological studies. Majority (90.00%) of cases, who were positive for Epstein Barr virus anti-IgG antibody, belonged to the younger age group. Firm diagnosis cannot be made on the basis of serology. This study proves that RT-PCR for EBV has to be interpreted along with serological antibody results. The qualitative RT-PCR for EBV needs not to come positive even with presence of EBV as a cause of cervical lymphadenopathy in paediatric patients, if the viral load is less. It also proves that serological antibody could be positive for EBV in many patients either because of cross reactivity with adenovirus or because of past infection with EBV.

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