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Detection of Human Cytomegalovirus in Patients with Meningitis by Using Real Time PCR

Ahmed Hasan Mohammed

ahmedhasan@sci.utq.edu.iq

Pathological Analysis Department, College of Science,Thi-Qar University, Nasiriya, Iraq

Abstract:

Background: Human Cytomegalovirus (HCMV) is one of the causes of meningitis and can affect the nervous system at all levels, from the hemispheres to the peripheral nerves, with presentations reflecting the pattern of anatomic involvement.**Aim of the study:** To assess the role of Human Cytomegalovirusin a cerebrospinal fluid (CSF) samples of Meningitis patientsusing Real time PCR method.**Materials and Method:** This study recruited Forty-EightMeningitis patients (referred as cases onward).The levels of glucose and proteins as well as cell cytology were used to roll out patients with other types of meningitis.DNA was isolated from CSF samples obtained from each participant. The HCMV UL36 gene was amplified with specific set of primers using Eva Green Real Time PCR.**Results:**results proved that 4(8.4%) out of 48 samples were real –time PCR positive the remainder 44 (91.6 %) were CMV real time PCR negative.**Conclusion:**Based on of these results, we concluded that HCMV should not be rule out as one of the most important causes of meningitis in Iraq.

Key Words: meningitis, CSF, human cytomegalovirus (HCMV), real time PCR

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الكشف عن الفايروس المضخم للخلايا البشري في المرضى الذين يعانون من التهاب السحايا بواسطة تفاعل البلمرة المتسلسل اللحظي

أحمد حسن محمد

ahmedhasan@sci.utq.edu.iq

قسم التحليلات المرضية – كلية العلوم – جامعة ذي قار – الناصرية – العراق

الخلاصة

يعتبر الفيروس المضخم للخلايا البشري Human Cytomegalovirus أحد أسباب التهاب السحايا ويمكن أن يؤثر على الجهاز العصبي. الهدف من الدراسة هو لتقييم دورهذا الفيروس في عينات سائل النخاع الشوكي من مرضى التهاب السحايا باستخدام طريقة تفاعل البلمرة المتسلسل اللحظي. تم جمع عينات سائل النخاع الشوكي من ثمان واربعين مريضا من مرضى التهاب السحايا الذين تم تشخيصهم في مستشفى مدينة الطب ومستشفى العلوية للأطفال في بغداد واستخدمت مستويات الجلوكوز والبروتينات وكذلك العد التفريقي لخلايا الدمللتفريق بين المرضى الذين يعانون من أنواع أخرى من التهاب السحايا. تم عزل الحمض النووي من عينات سائل النخاع الشوكي التي تم الحصول عليها من كل مريض وتم تضخيم الجين UL36 الخاص بالفايروس باستخدام مجموعة محددة من البادئات Primers وباستخدام تفاعل البلمرة المتسلسل اللحظي ذو صبغة إيفا الخضراء Eva Green Real Time PCR. أظهرت النتائجأن 4 (8.4٪) من أصل 48 عينة كانت موجبة والباقي 44 (91.6٪) كانت سالبة وان بقية العلامات الخاصة بسائل النخاع الشوكي مثل العدد التفريقي للخلايا ونسبة الكلوكوز والبوتين تشير الى وجود التهاب سحايا من النوع الفايروسي.
استنادا إلى هذه النتائج، خلصنا إلى أن الفايروس المضخم للخلايا البشري لا ينبغي أن يكون خارج توقعات اصابته للسحاياواعتباره احدمسببات التهاب السحايا في العراق.

الكلمات الرئيسية: التهاب السحايا، سائل النخاع الشوكي، الفيروس المضخم للخلايا البشري،تفاعل البلمرة المتسلسل اللحظي.

Introduction

Human Cytomegalovirus (HCMV) is the prototype member of Betaherpesvirinae in the subfamily Herpesviridae. The structure of HCMV is similar to other members of Herpesviridae. It is an enveloped virus with icosahedral symmetry containing a large genome of double stranded DNA. HCMV has the largest genome of around 235 kb encoding for over 200 genes (1).

HCMV has a longer replication cycle and it encodes for a diverse array of gene products, many of which play an immunomodulatory role in the host. Its genome codes sequentially for three genes, which encode for immediate early, early and late proteins respectively. The immediate early (IE) proteins are regulatory and the late proteins are structural. Many late proteins such as pp65 antigen have antigenic properties which can be used for diagnosis of HCMV infection (2).

Primary HCMV infection is not a problem in those critically ill immunocompetent patients. The most common mechanism proposed for HCMV infection or disease in such critically ill patients is because of reactivation of HCMV virus and not primarily due to primary infection. HCMV has the ability to establish latency in various different types of cells unlike other herpes viruses which largely remain localized (2,3).

HCMV is endemic in most areas of the world. The seroprevalence of HCMV varies in different geographical areas and it ranges from 30-100% (1, 4).This virus is responsible for many fetal defects, neonatal inborn malformations and various syndromes in children and adults. The clinical course of HCMV infection is mostly asymptomatic. Subclinical courses of the infection, a mononucleosis-like syndrome and disseminated HCMV infections have also been reported(5).

Herpes simplex virus (HSV)-1, HSV-2, varicella-zoster virus (VZV), Ebstein-Barr virus (EBV), cytomegalovirus (HCMV), and human herpesvirus-6 collectively cause approximately 4% of cases of viral meningitis, with HSV-2 being the most common offender. The viruses may attack at any time of the year. When associated with encephalitis, however, the mortality rate can be high. Early treatment with acyclovir can significantly reduce morbidity. Viral pathogens may gain access to the central nervous system (CNS) via either of two main routes: hematogenous and neural. The hematogenous route is more common for penetration of most known viral pathogens. Neural penetration refers to spread along nerve roots and is usually limited to herpes viruses (6).

HCMV can affect the nervous system , from the hemispheres to the peripheral nerves, with presentations reflecting the pattern of anatomic involvement. Clinically, the patient can present with a febrile encephalopathy, myelopathy, optic neuropathy, psychosis, hallucinations, hemiplegia with headache, brainstem involvement, locked-in syndrome—the entire panoply of neurologic syndromes. CNS manifestations are very common in immunocompromised patients. However, there have been

reports of meningitis and other CNS manifestations as a result of HCMV even in immunocompetent patients(7, 8).

The aim of this study was to investigatethe existenceof HCMV in the CSF sample of patients with meningitis.

Subjects and Materials

Subjects:

Cerebrospinal fluid specimens obtained from Forty-eight (48) patients suspected with central nerve system infections and who admitted to Baghdad City Hospital and Al-Elwia pediatrics Hospital in Baghdad.The patients were divided according to gender into twenty-eight (28) males and twenty (20) females (n=48) their age range was from 1 to 35 years old. Patient's selection based on clinical and laboratory criteria including; the levels of glucose and proteins as well as cell cytology to roll out patients with other types of meningitis. Five to nine milliliters of CSF samples were collected in sterile containers and separate into two tubes, one processed to examine the appearance and measureof protein, sugar,and cell count and the another tube was stored in – 20 ºC for DNA extractions.

Biochemical Tests:

Protein detection: the concentration of protein was detected in the CSF samples of meningitis using Biuret method. This method depends on the detection of peptide bonds. The intensity of the color at 540 nm is directly proportional to the concentration of the protein according to the Beer-Lambert law.

Glucose detection: the concentration of glucose was detected in the CSF samples of meningitis using biochemical method. Glucose Enzyme Mix specifically oxidizes glucose to generate a product which reacts with a dye to generate color measured at 570 nm. The generated color is proportionally to the glucose amount.

Genomic DNA extraction and Real time PCR:

DNA was extracted from CSF samples using DNA isolation kit (DNA-sorb-B (Sacace) /Italy) Kit) according to the manufacturer's instruction.The concentration and purity of the purified DNA was quantified by the use of nanodrop instrument following the instruction of the manufacturer,Purified DNA rehydrated by rehydration solution and store at -20 ºC.

DNA of HCMV was detected using Eva Green with dissociated curve real time PCR procedure. The specific pair of primer was used for anti-apoptotic, Unique long (UL36) gene. The sequences of the primers were as follows:

HCMV-UL36 forward primer 5'-CTCGTGCGTCATCACGTTTC-3' and HCMV-UL36 reverse primer 5'-TCCACACTATGCGCGACTAC-3'. Briefly, the first round of PCR was carried out in 20 µl total volume containing 0.5 µl of each forward and revers primers (10 pmol/µl), 5 µl of extracted DNA used as template, 4 µl of Hot Tag Eva Green master mix and 10 µl of nuclease free H2O. Reaction conditions included an initial denaturation step at 95 ºC for 15 minutes, followed by 40 cycles of 95 ºC for 15 seconds, 60 ºC for 60 seconds and 72 ºC for 20 seconds. All PCR amplifications were conducted using sterilized tubes and tips in a biosafety hood that was not previously exposed to HCMV. Controls without HCMV template tested negative affirming the lack of contamination.During this process fluorescence was continuously monitored and melting curves were constructed automatically then converted to melting peaks. Before starting a real time PCR reaction choose Eva Green filter for unknown sample and ROX low for standard. The standard curve was constructed using a 10-fold dilution by plotting the log of starting quantity of template against the CT value obtained during amplification of each dilution. Real-time PCR was performed using Agilent Real-time PCR (Techne-UK).

Ethical Approval

This research underwent to the terms of ethical considerations and in accordance with the form prepared for this purpose by the Iraqi Ministry of Health also got the approval of the research by the Committee of ethical standards in the Faculty of Medicine, Al-Nahrain University, one of the colleges affiliated to the Ministry of Higher Education and Scientific Research, Iraq.

Results

From the whole study populations 20 were females (41.6%) and 28 were males (58.3%), there was a no statistical significant difference between age and sex distribution, ratio of males to females 1.1:1.It was found that among 4 positive cases with HCMV meningitis females were 3(75%) and only one male (25%) Table (1):

Table (1): Age and gender distribution among patients with Study group.

Age groups	Frequency (%)	Male	Female
<10 years	3 (6.25)	2	1
10-19 years	4 (8.33)	4	0
20-29 years	10 (20.8)	3	7
30-39 years	6 (12.5)	4	2
40-49 years	11 (22.91)	5	6
50-59 years	10 (20.8)	8	2
≥ 60 years	4 (8.33)	2	2
Total	48 (100)	28 (58.3%)	20 (41.6%)

Macroscopic observations

The samples of CSF were tested macroscopically for appearance and color and the results showed 33 (68.7%) samples were clear, 11 (20.7%) samples were turbid and 5 (10.4%) samples were yellow in color.

Biochemical analysis and WBC count:

Cell count and differentiation were done on all CSF samples (n=48), the mean of white blood cells was (420 cell/cmm) and predominantly, lymphocytic pleocytosis with a mean of (74%) versus (26% for neutrophil) The average volume of CSF was 5-9 ml, the mean of glucose level was (43.51 mg/dl) and mean of protein level was (150.30 mg/dl), table (2). Analysis of the subgroup of patients that were subsequently proved to be real- time PCR positive for HCMV meningitis (n=4) displayed typical changes in CSF parameters with elevated white blood cell predominantly lymphocytes as well as high level of protein with low level of sugar.

Table (2): Biochemical tests and WBC count in CSF samples.

Test	Patients	Normal value
	Mean	Range
CSF volume	5-8 ml	Not applicable

WBC total count	420 cell/mm^3	0 - 5 cell/mm^3
Neutrophil %	26%	0% - 6 %
Lymphocytes %	74%	40% - 60%
Glucose mg/dl	43.51mg/dl	40 - 70 mg/dl
Protein mg/dl	150.30mg/dl	15 - 40 mg/dl

Results of real time PCR

The results of real time PCR showed detection of HCMV UL36 gene in four samples (8.4%) from 48 samples of CSF.The mean of viral load was equal to $3.772*10^3 \pm 1.965*10^3$ copy number/ml and median of threshold cycle (CT) equal to 37.19 from 50 cycle reaction. The efficiency of real time PCR reaction was about 96.2% (figure 1).This result was compatible with typical changes in CSF parameters with elevated white blood cell predominantly lymphocytes as well as high level of protein with low level of sugar.

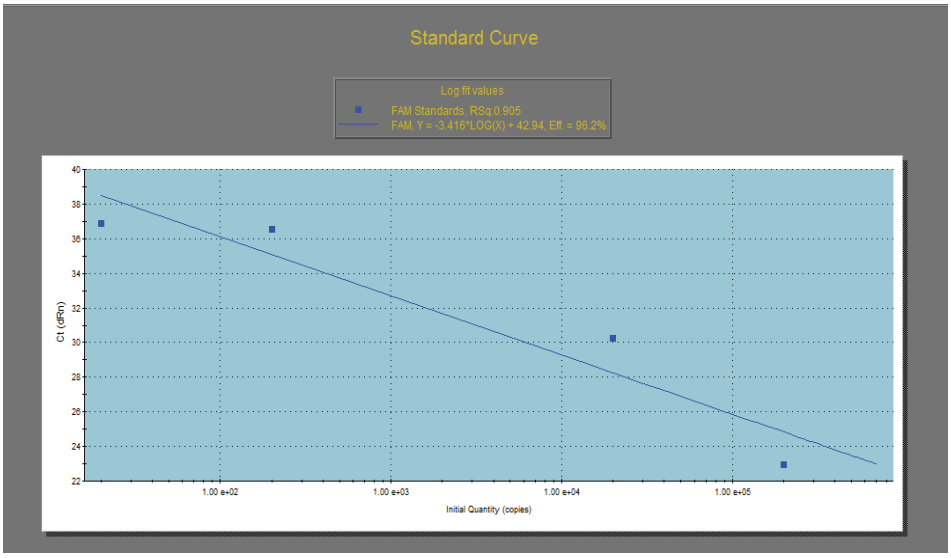

Figure (1): Standard curve of real time PCR showing the efficiency of reaction. X-axis represents CT number and Y-axis represents initial quantity (copies)

Discussion

The difficulty and warning of obtaining CSF samples were the reasons behind the lack of control samples in the current research, so that rely solely on patient's samples in the diagnosis of the presence of HCMV and investigate its probability to cause meningitis.In developed countries as

many as 60–80% of the population will be infected with HCMV by adulthood but its actual prevalence rate in Iraq remains unknown (9). Suggesting that HCMV meningitis should be included in the differential diagnosis of immunocompetent adults with lymphocytic meningitis. Treatment of HCMV meningitis in patients that are not immunocompromised is debatable. Prospective studies will be needed to clarify this issue further. The risk ratio oftoxicity treatment against the risk of neurological deterioration due to HCMV meningitis mandates the administration of ganciclovir (7).

In the majority of persons, primary HCMV infection is clinically asymptomatic. When symptomatic, HCMV disease in immunocompetent persons may present as mononucleosis syndrome or as flu-like symptoms (10).The virus can spread by horizontal transmission (direct contact with virus containing secretions) or by vertical transmission via transplacental rout (11).HCMV can be latent in the central nervous system, then the absence of serum anti-HCMV antibody with presence of HCMV in CSF should be indicated of local CNS reactivation. The most sensitive method to detect HCMV in the blood or other body fluids may be by PCR because anti-HCMV antibody may stay in serum for more than one year after infection (12).In this study CSF profile was significantly abnormal in definite diagnosis and in all cases that have been studied. CSF picture revealed high level of protein and low level of sugar and white blood cell was high in number with predominance lymphocytic pleocytosis. This indicates that each of cell count and chemical picture of CSF were offers a good diagnostic prediction in patients with viral meningitis.

The biochemical analysis of CSF including high protein level, low glucose level, and high percentage of lymphocytes indicate viral meningitis but not to specific type of virus. In viral neuroinfectious, CSF protein concentrations are raised to a lesser degree (usually < 0.95 g/l) (13). An increased number of neutrophilic granulocytes canbe found in bacterial and acute viral CNS infections (14,15).

Many studies indicate that the sensitivity of real-time PCR is very high when compared with conventional or single plex PCR in addition to the significant advantage in time saving and avoidance of the risk of cross-contamination and measuring of viral load that characterize the real time PCR (16,17).

The result of real time PCR showed low percentage of HCMV gene detection in the CSF samples and this result either real and indicates the actual percentage of HCMV meningitis among other types of viral meningitis or the viral load was under the detection limit of the real time PCR materials so that. However, the association between HCMV and meningitis is under investigation and although there was strong evidence on viral meningitis caused by members of herpesviridae like EBV and HSV but rare studies indicate to the association between HCMV and meningitis.

Many case reports had been published refereeing to HCMV meningitis (7, 8). It is not clear whether HCMV meningitis is a self-limited disease or a disease with potential catastrophic consequences for the patient (18).

Most of the laboratory results of the CSF samples in this study showed a features of aseptic meningitis hence it could be possible a viral cause and since HCMV is widely latent on human, then the four cases could be HCMV meningitis. Moreover, the rest of the cases (44 samples) could have HCMV meningitis but the detection level of the kit used in real-time PCR may be below the detection level of the available HCMV DNA.

Conclusion

The presence of HCMV in CSF sample of patients with meningitis although in low percentage indicate to the risk of prevalence of HCMV and its opportunity to reach blood stream and CSF then causing serious disease like meningitis. On the other hand, the results of this study indicate and confirm the ability of HCMV to cause neurological manifestations not only in fetus and also in children with competent immunity. This study suggest that HCMV meningitis should be included in the differential diagnosis of immunocompetent patients with lymphocytic meningitis. Prospective studies might be needed to clarify this issue further.It is clearly seen that the authority of the risk of treatment-associated toxicity against the risk of neurological deterioration due to HCMV meningitis mandates the administration of ganciclovir.

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